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# Behavioral, molecular and electrophysiological characterization of the learning and memory deficits induced in mouse models of Alzheimer's disease

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**L'UNIVERSITÉ DE BORDEAUX**

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Spécialité : Neurosciences

Par **Senka HADZIBEGOVIC**

**Behavioral, molecular and electrophysiological characterization of  
the learning and memory deficits induced in mouse models of  
Alzheimer's disease**

Sous la direction de: Bruno BONTEMPI

Soutenue le 10 Septembre 2015

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## **Caractérisations comportementales, moléculaires et électrophysiologiques des déficits mnésiques induits à l'aide de modèles murins de la maladie d'Alzheimer**

### **Résumé**

La maladie d'Alzheimer (MA) se caractérise par une perte des fonctions cognitives liée à une dégénérescence neuronale induite par l'accumulation de peptides amyloïdes- $\beta$  (A $\beta$ s) dans des régions vulnérables du cerveau comme l'hippocampe. Au niveau moléculaire, les peptides A $\beta$ s se lient préférentiellement à la densité post-synaptique des synapses excitatrices, espace au niveau duquel la protéine d'échafaudage PSD-95 organise l'ancrage des récepteurs NMDA (RNMDAs) et régule leur mobilité membranaire. A l'aide d'une stratégie intégrative qui favorise des niveaux d'analyse verticaux (du phénotype aux événements moléculaires) et qui combine un ensemble d'approches corrélatives et invasives chez des souris double transgéniques APPswe/PS1dE9 modèles de la MA, nous avons mis en évidence que les peptides A $\beta$ s déstabilisent l'organisation synaptique (altération de l'expression de la PSD-95) et augmente le pool extrasynaptique de sous-unités GluN2B des RNMDAs dans l'hippocampe. Cette réorganisation se traduit par une perturbation des fonctions mnésiques. Par ailleurs, il a été montré que certaines oscillations de l'activité hippocampique, comme les « sharp-wave ripples » (SWRs) générées pendant les périodes de sommeil, jouent un rôle crucial dans la formation de la mémoire. De façon surprenante, l'accumulation des peptides A $\beta$ s semblent épargner la dynamique d'expression des SWRs durant les comportements de routine. Afin d'examiner l'effet potentiel des A $\beta$ s sur les SWRs chez des animaux confrontés à des challenges cognitifs, nous avons soumis des souris adultes injectées intracérébralement avec une solution d'A $\beta$ s à un test de reconnaissance spatiale. Alors qu'elles sont capables de former une mémoire à court terme, les souris A $\beta$ s montrent un oubli plus rapide, suggérant qu'elles encodent avec succès, mais qu'elles sont incapables de stabiliser et de rappeler une information acquise antérieurement. En l'absence d'une demande cognitive préalable, les propriétés des SWRs ne sont pas altérées par les A $\beta$ s. En revanche, lorsqu'elles doivent résoudre un test cognitif, les pics de SWRs normalement observés après encodage ou reconnaissance chez les souris témoins sont abolis chez les souris A $\beta$ s, indiquant une perturbation du traitement hippocampique de l'information spatiale. Pris dans leur ensemble, ces résultats identifient deux nouveaux mécanismes délétères sous-tendant les déficits de mémoire spatiale associés à la MA.

Mots clés: mémoire spatiale, récepteurs NMDA, maladie d'Alzheimer, oscillations hippocampiques, souris transgéniques

### **Behavioral, molecular and electrophysiological characterization of the learning and memory deficits induced in mouse models of Alzheimer's disease**

#### **Summary**

Cognitive impairments in Alzheimer's disease (AD) are thought to be related to degenerative synaptic changes caused by the accumulation of amyloid- $\beta$  peptides (A $\beta$ s) in vulnerable brain regions such as the hippocampus. At the molecular level, A $\beta$ s bind preferentially to the postsynaptic density of neuronal excitatory synapses, where the scaffolding post-synaptic protein-95 (PSD-95) organizes NMDA receptor (NMDAR) location as well as its downstream signaling. By using an integrative strategy which favoured vertical levels of analyses (from phenotype to molecular events) and combined a set of interrelated correlative and invasive approaches in a double transgenic mouse model of AD (APPswe/PS1dE9 mice), we were successful in establishing that A $\beta$ s destabilize the synaptic organization (reduction of expression of PSD-95) and increase the extrasynaptic pool of GluN2B-containing NMDAR in the hippocampus, a reorganization which translates into impaired memory functions. It is also well-known that hippocampal sharp wave-ripples (SWRs) generated during sleep periods are crucial for memory formation but accumulation of soluble A $\beta$ s, surprisingly seems to spare SWR dynamics during routine behavior. To unravel a potential effect of A $\beta$ s on SWRs in cognitively-challenged animals, we submitted vehicle- and A $\beta$ -injected mice to spatial recognition memory testing. While capable of forming short-term memory, A $\beta$  mice exhibited faster forgetting, suggesting successful encoding but an inability to adequately stabilize and/or retrieve previously acquired information. Without prior cognitive requirements, similar properties of SWRs were observed in both groups. In contrast, when cognitively challenged, the post-encoding and -recognition peaks in SWR occurrence observed in controls were abolished in A $\beta$  mice, indicating impaired hippocampal processing of spatial information. Altogether these results identify two new disruptive mechanisms for the spatial memory deficits associated with AD.

Keywords: spatial memory, NMDA receptors, Alzheimer's disease, hippocampal oscillations, transgenic mice

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# PUBLICATIONS AND COMMUNICATIONS

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- **O.Nicole\***, **S.Hadzibegovic\***, J.Gajda, B.Bontempi, T. Bem, P. Meyrand. Alterations of learning-induced hippocampal ripples in a mouse model of Alzheimer's disease impair spatial memory formation. (*submitted*) \*Contributed equally
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**S.Hadzibegovic**, Y.Cho, N.Macrez, B.Bontempi, O.Nicole.
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O.Nicole\*, **S.Hadzibegovic\***, J.Gajda, B.Bontempi, T. Bem, P. Meyrand.

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**S.Hadzibegovic**, Y.Cho, S.Pietropaolo, B.Bontempi, O.Nicole.

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**S.Hadzibegovic**, Y.Cho, S.Pietropaolo, B.Bontempi, O.Nicole.

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## LIST OF ABBREVIATIONS

**AICD:** amyloid precursor protein Intracellular domain  
**AMPA(R):**  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)  
**APOE:** apolipoprotein E  
**APP:** amyloid precursor protein  
**ATD:** amino-terminal domain  
**A $\beta$ :** amyloid beta  
 **$\alpha$ 2M:** alpha 2-macroglobulin  
 **$\alpha$ 7 nAChR:** alpha 7 nicotinic acetylcholine receptor  
**BACE:** beta-site amyloid precursor protein cleaving enzyme  
**BBB:** blood-brain barrier  
**BDNF:** brain-derived neurotrophic factor  
**CAA:** cerebral amyloid angiopathy  
**CaMKII:** Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  
**CREB:** cyclic-adenosine monophosphate response element binding protein  
**CTD:** carboxyl-terminal domain  
**CTF $\alpha$ / $\beta$ :** carboxyl-terminal fragment  $\alpha$ / $\beta$   
**EAAT1-5:** excitatory amino acids transporters 1-5  
**ER:** endoplasmic reticulum  
**ERK 1/2:** extracellular signal regulated protein kinase 1/2  
**Extra-NMDARs:** extrasynaptic NMDARs  
**GABA:** gamma-aminobutyric acid  
**GLAST:** glutamate aspartate transporter  
**GLT-1:** glutamate transporter-1  
**IP3:** inositol triphosphate  
**KPI:** kunitz-type protease inhibitor domain  
**LTD:** long-term depression  
**LTP:** long-term potentiation  
**MAGuK:** membrane-associated guanylate kinases  
**NEP:** neprilysin  
**NMDA(R):** N-methyl-D-aspartate (receptor)  
**PS:** presenilin  
**PSD:** postsynaptic density  
**SAP 102/97:** synapse-associated protein 102/97  
**sAPP $\alpha$ / $\beta$ :** soluble N-terminal fragment of APP  $\alpha$ / $\beta$   
**SWS:** slow wave sleep  
**SWR:** sharp-wave ripple  
**Syn-NMDARs:** synaptic NMDARs

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# GENERAL INTRODUCTION

# I. NMDA RECEPTORS

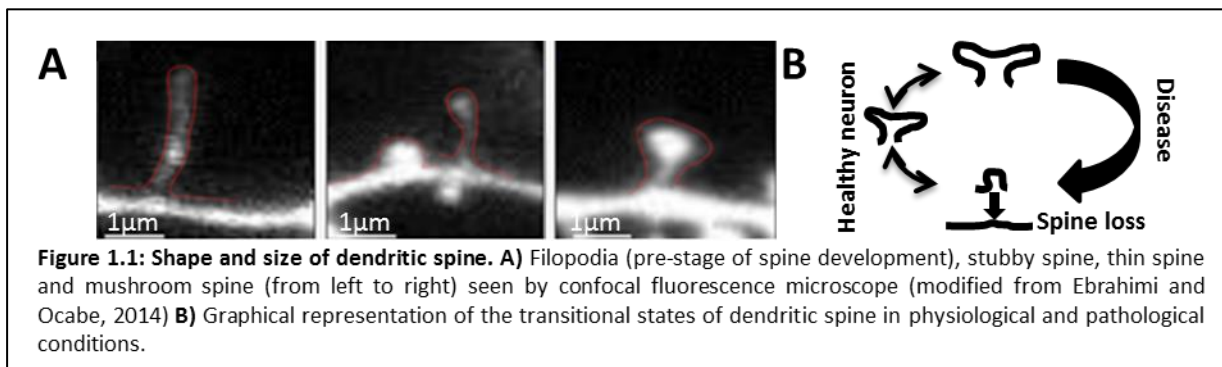


## A. Glutamatergic excitatory neurotransmission: overview

### 1. Structural organization of excitatory synapse

#### a) Spine structure

Most excitatory synapses in the brain are built on spines, small protrusions of a neuron's dendrite membrane that receive inputs from presynaptic neurons. **Spines constitute a specialized compartment that contains the postsynaptic signaling machinery, and organelles such as endosomes** (Kennedy and Ehlers, 2006). Many studies indicate that a mutual relationship exists between spine morphology and function of synapses (Alvarez and Sabatini, 2007; Bourne and Harris, 2008), and that the actin cytoskeleton which composes a spine has a critical role in modulating the efficacy of pre- and postsynaptic terminals (Cingolani and Goda, 2008; Frost et al., 2010). According to their shape and size, spines can be classified into three classes: thin, stubby and mushroom. These three classes do not constitute special entities but rather illustrate the different transition states that a spine can take (Figure 1.1).



Live-cell imaging reveals that spines are very dynamic structures that undergo reversible transition from one state to another between few minutes and hours. This morphological remodeling occurs mostly during development and in adaptation to sensory stimuli or in learning and memory. Indeed, the size and the shape of the spine neck and head have been shown to play an important role in synaptic signaling and synaptic strength (Noguchi et al., 2005). Although diverse in size and morphology, all classes of spines contain the following general components: post-synaptic density area (PSD), actin cytoskeleton and soluble regulatory proteins.

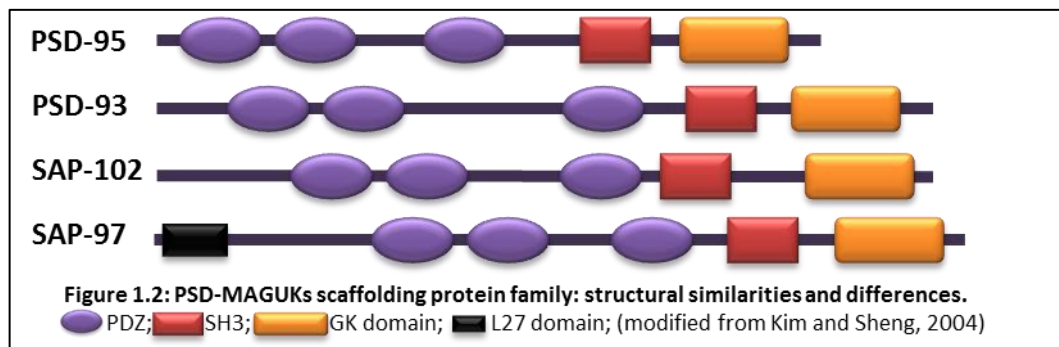
### ***b) The post-synaptic density or PSD***

The postsynaptic density area (PSD) of spines, located just below the plasma membrane, is a disk-like structure  $\approx 30\text{--}40$  nm thick and up to a few hundred nm wide. It is relatively insoluble in nonionic detergents and can be purified to a considerable degree by differential centrifugation (Carlin et al., 1980). It has been proposed that PSD is a crucial place where organization of neurotransmitter receptors occurs (Ziff, 1997) by protein–protein interaction and interaction with the actin cytoskeleton (Rosenmund and Westbrook, 1993).

PSDs are composed of a dense network of proteins that can be divided into several classes: cell-adhesion proteins, cytoskeletal proteins, scaffolding and adaptor proteins, membrane-bound receptors and channels, G-proteins and modulators and signaling molecules including kinases/phosphatases (Sheng, 2001). The core of this specific organization is mainly related to the scaffold proteins. The scaffold protein, such as the members of the PSD-95-like membrane associated guanylate kinases (MAGuKs) or SH3, ankyrin repeat-containing protein (Shank/ProSAP) family (Lim et al., 1999), guanylate kinase-associated protein (GKAP/SAPAP) and Homer (Tao-Cheng et al., 2014) are all components of the postsynaptic density area. While without intrinsic enzymatic activities, they are crucial regulators of many synaptic functions including the trafficking, anchoring, and clustering of glutamate receptors and adhesion molecules and in that way, they regulate the signaling pathways and the dynamics of cytoskeletal structures (Boeckers, 2006).

Particularly, PSD-MAGuKs form a major family of scaffold proteins at glutamatergic synapses, including PSD-95 (SAP90), PSD-93 (Chapsyn-110), SAP102 and SAP97. They all share a common structure composed of three PSD-95/Discs large/Zona occludens-1 (PDZ) domains, followed by a Src-homology-3 (SH3) domain and a catalytically inactive guanylate kinase (GK) domain (Figure 1.2). PDZ are modular protein-interaction domains that typically bind specific sequences in target proteins: a carboxyl-terminal hydrophobic residue and a free carboxylate group, as exemplified by the E(S/T)DV motif of certain ion channel subunits (Kim and Sheng, 2004). SH3 domains bind proline-rich peptide sequences with the consensus PXXP (Feng et al., 1994). Serine phosphorylation of a PDZ or SH3 recognition site results in uncoupling of target proteins. The roles of the scaffold proteins within PSDs are numerous.

They regulate the relative number and positioning of glutamate receptors subunits. They are involved in intracellular trafficking of the receptors, in recycling processes as well as in the endocytosis and exocytosis of these receptors. PSD scaffolds may also control the receptor's targeting on the cell surface of dendritic spine, a process which can be a crucial step in the establishment of long lasting changes in synaptic transmission (Funke et al., 2005; Iasevoli et al., 2013).



## 2. Glutamate as a neurotransmitter

The functional implication of glutamate in the central nervous system (CNS), aside from its obvious one as a protein constituent, was first considered more in terms of energy metabolism, given the close association of amino acids with the Krebs cycle. However, an early indication of a special role of glutamate in electrophysiological processes was introduced by Hayashi (1952) when observing that injections of glutamate into the brain or carotid arteries could produce convulsions. He speculated that glutamate was a transmitter in the mammalian CNS (Hayashi, 1952). It was not until the late 1970s, however, that glutamate became widely recognized as a principal excitatory amino acid neurotransmitter abundantly present in the brain of mammals. As a major excitatory neurotransmitter, **released by an estimated 40% of all synapses, glutamate is indispensable and involved in most aspects of normal brain functions** including behavior, cognition, movement, sensation and in the formation of neural networks during development. Because of its ubiquitous role as a neurotransmitter, it follows that disruptions in the normal functioning of glutamatergic signaling can have severe detrimental effects on the CNS. A major source of cellular stress is related to glutaminergic overstimulation of a postsynaptic neuron, a process known as excitotoxicity, which was first discovered by Lucas and Newhouse in 1957 during experimentation on monosodium glutamate-fed neonate mice (Lucas and Newhouse, 1957).

### 3. **Glutamate release and uptake**

Glutamate is released from vesicles in presynaptic terminals via a  $\text{Ca}^{2+}$ -dependent mechanism that involves N- and P/Q-type voltage-dependent  $\text{Ca}^{2+}$  channels (Birnbaumer et al., 1994) and which appears to be closely linked to vesicle docking sites. The glutamate concentration within the vesicle is thought to be  $\sim 100$  mmol/L. The synaptic release of glutamate is controlled by a wide range of presynaptic receptors (glutamatergic and cholinergic receptors, adenosine A1, kappa opioid,  $\gamma$ -aminobutyric acid ( $\text{GABA}$ )<sub>B</sub>, cholecystinin and neuropeptide Y receptors (Meldrum, 1998).

Glutamate, as all amino-acid and monoamine neurotransmitters, possesses specific, **high-affinity transport mechanisms** designed to terminate the synaptic actions of the neurotransmitter and to recycle the molecules involved. Extracellular glutamate concentrations in the CNS are regulated by a family of high-affinity,  $\text{Na}^+$ -dependent glutamate transporters. Five subtypes of transporters, EAAT1–EAAT5 (excitatory amino acids transporters 1–5), have been identified, two of which, the glutamate transporter GLT-1 (also known as EAAT-2) and the glutamate-aspartate transporter GLAST (also known as EAAT-1 or GLT-2), are predominantly present on astrocytes and are the major glutamate transporters in the CNS (Beart and O'Shea, 2007). Of the bulk of **glutamate released during synaptic transmission, about 20% is accumulated in postsynaptic neurons and the remaining 80% is taken up by perisynaptic astrocytes through glutamate transporters** (Verkhratsky and Kirchhoff, 2007). Malfunction or aberrant expression of these glutamate transporters can cause accumulation of toxic concentrations of glutamate and trigger neurodegeneration. Reduced GLT-1 protein expression occurs in brain injury or ischemia, Alzheimer's disease, Huntington's disease, HIV-1-associated dementia, and experimental autoimmune encephalomyelitis (Kaul et al., 2001; Shigeri et al., 2004; Rossi et al., 2007; Mitosek-Szewczyk et al., 2008).

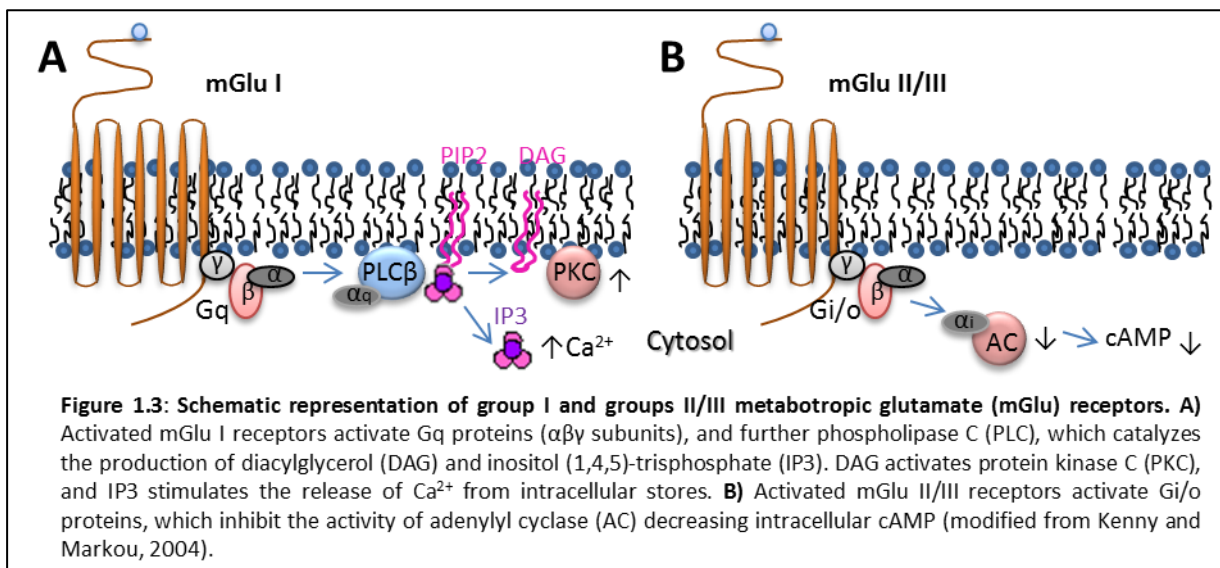
### 4. **Glutamatergic receptors**

The central effects of glutamate were thought to be exclusively mediated by ion channel mechanisms. Based on pharmacological and molecular approaches, glutamate receptors (GluRs) can now be categorized into two groups: ionotropic (iGluR) and metabotropic (mGluR). The iGluRs are ligand-gated ion channels, mainly localized

postsynaptically, and are characterized by their selective affinity for the following specific agonists: **NMDA** (N-methyl-D-aspartate), **AMPA** ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and **kainate**. In contrast, mGluRs, which are frequently present in the presynaptic membrane, do not form ion channels but are associated to G proteins and coupled to the production of second intracellular messengers.

### a) *Metabotropic receptors*

Glutamate metabotropic receptors, which share a common morphology with other G-protein linked receptors and are insensitive to AMPA, NMDA and kainate, have been identified and classified into three groups according to their sequence homology and their coupling to a second messenger. The activation metabotropic receptors belonging to group I (mGluR1 and mGluR5) activates phospholipase C causing the hydrolysis of phosphatidylinositol bisphosphates (PIP<sub>2</sub>) and the release of diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>) as second messengers. Further, it leads to the release of Ca<sup>2+</sup> from intracellular stores in the endoplasmic reticulum. Specific agonists of group II (mGluR2, mGluR3) and group III (mGluR4, mGluR6, mGluR7, mGluR8) lead to the activation of an inhibitory G protein, which negatively regulates the activity of adenylate cyclase and further decrease the amount of cyclic adenosine monophosphate (cAMP) in the cell (Conn and Pin, 1997; Meldrum, 2000) (Figure 1.3).

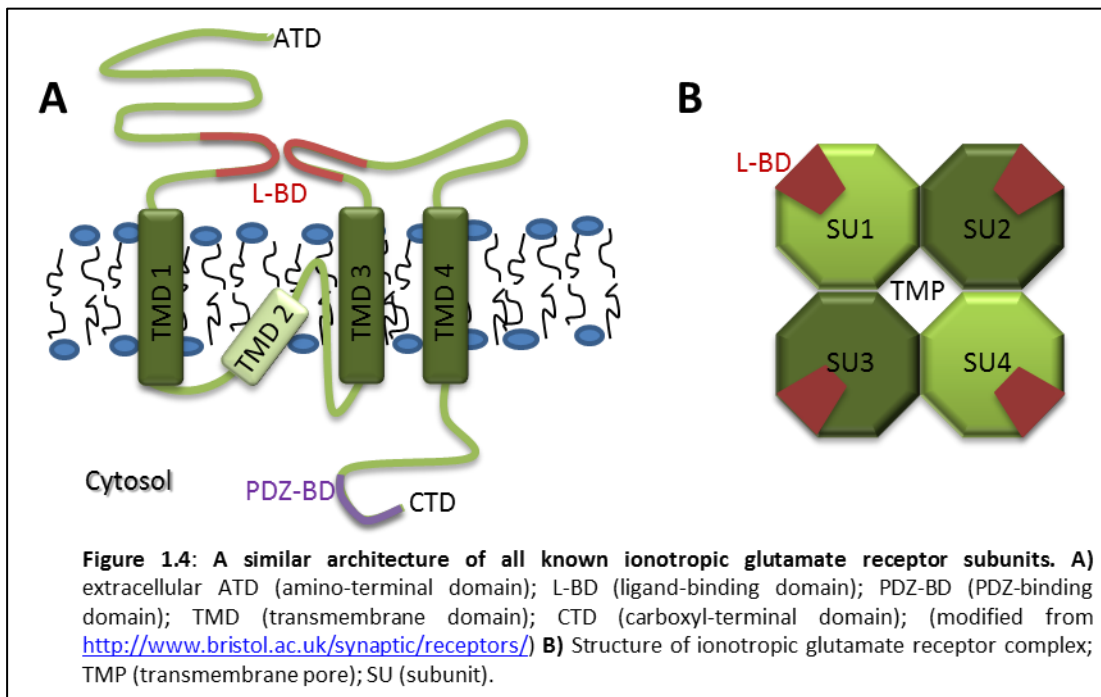


Metabotropic receptors from group I are expressed in the striatum at the level of glutamatergic, GABAergic and dopaminergic post-synaptic endings (Smith et al., 2000; Kew

and Kemp, 2005). The groups II and III are preferentially localized at the presynaptic level where they modulate the release of glutamate and other neurotransmitters co-released with glutamate (Cartmell and Schoepp, 2000).

### **b) Ionotropic receptors**

The mammalian ionotropic glutamate receptor family is encoded by 18 genes. Gene products co-assemble to form ligand-gated ion channels containing an agonist recognition site, a transmembrane ion permeation pathway, and gating elements that couple agonist-induced conformational changes to the opening or closing of the permeation pore. Ionotropic glutamate receptors mediate fast excitatory synaptic transmission in the CNS and are integral membrane proteins composed of four large subunits (>900 residues) that form a central ion channel pore. Their central pore is permeable to sodium ions ( $\text{Na}^+$ ), calcium ( $\text{Ca}^{2+}$ ) and potassium ( $\text{K}^+$ ). Sequence similarity among all known glutamate receptor subunits, including the AMPA, kainate and NMDA receptors, suggests that they share a similar architecture. Glutamate receptor subunits are modular structures that contain four discrete semiautonomous domains: the extracellular amino-terminal domain (ATD), the extracellular ligand-binding domain (LBD), the transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD) (Traynelis et al., 2010) (Figure 1.4).



The AMPAR subunits GluA1 to GluA4 can form both homo- and heteromers. The kainate receptor subunits GluK1 to GluK3 also form both homo- and heteromers, but GluK4 and GluK5 form functional receptors only when coexpressed with GluK1, GluK2 or GluK3 (Carta et al., 2014). Functional NMDARs require assembly of two GluN1 subunits together with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Monyer et al., 1994; Traynelis et al., 2010).

From a physiological point of view, there are significant difference in kinetics between AMPARs/kainate receptors and NMDARs. AMPA and kainate receptors are rapidly activated by high concentrations of glutamate with a high probability of opening. Since they possess low-affinity binding sites for the glutamate, they have fast deactivation kinetics. Gating of AMPA and kainate receptors by glutamate is extremely fast in contrast to the slow gating of NMDARs. This rapid activation and short opening time of **AMPARs facilitate unblock of slow-activating NMDARs by Mg<sup>2+</sup> and therefore enables participation of these NMDARs in the regulation of synaptic currents** (Dingledine et al., 1999) (Figure 1.5).

	AMPA	NMDA	KA
<b>Receptor complex nature</b>	Homo-/Hetero-mer	Heteromer	Homo-/Hetero-mer
<b>Subunits</b>	GluA1-4	GluN1-2(ABCD)-3(AB)	GluK1-5
<b>Permeability</b>	Ca <sup>2+</sup> , Na <sup>+</sup> , K <sup>+</sup>	Na <sup>+</sup> , Ca <sup>2+</sup> , K <sup>+</sup>	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup>
<b>Particularities</b>		TMP blocked by Mg <sup>2+</sup>	
<b>Localization</b>	Pre-/post-synaptic	Postsynaptic	Pre-/post-synaptic

**Figure 1.5: Characteristics and particularities of ionotropic glutamate receptor complexes** (TMP-transmembrane pore).

## B. NMDARs: properties and functions

### 1. Generality

NMDARs are ionotropic glutamate receptors composed of two GluN1 subunits and two GluN2 or GluN3 subunits. NMDARs are permeable to Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> but calcium permeability is thought to be the critical factor for many of the physiological and

pathological effects mediated by NMDARs. Indeed, increase in intracellular calcium concentration after NMDAR activation is necessary for some forms of synaptic plasticity which modify synaptic efficacy and neuronal morphology. For NMDARs to be activated, glutamate needs to bind to GluN2 subunits, glycine or D-serine to GluN1 subunits (Sanz-Clemente et al., 2013a) and the  $Mg^{2+}$  blockade needs to be released by membrane depolarization (Verdoorn et al., 1987). Beyond the glutamate and D-serine/glycine binding site, NMDARs contain several regulatory sites sensitive to polyamines,  $Zn^{2+}$ , protons, and glutathione (Dingledine et al., 1999). Seven different NMDAR subunits have been identified: GluN1, GluN2A-D and GluN3A-B. GluN1 is an essential component found in all tetramers, while different GluN2s are incorporated based on the developmental stage (newborn versus adult), their, the localization and the distribution in either CNS. **Many of functional properties of NMDARs may rely on their subunit composition, localization and distribution.**

## 2. Genes

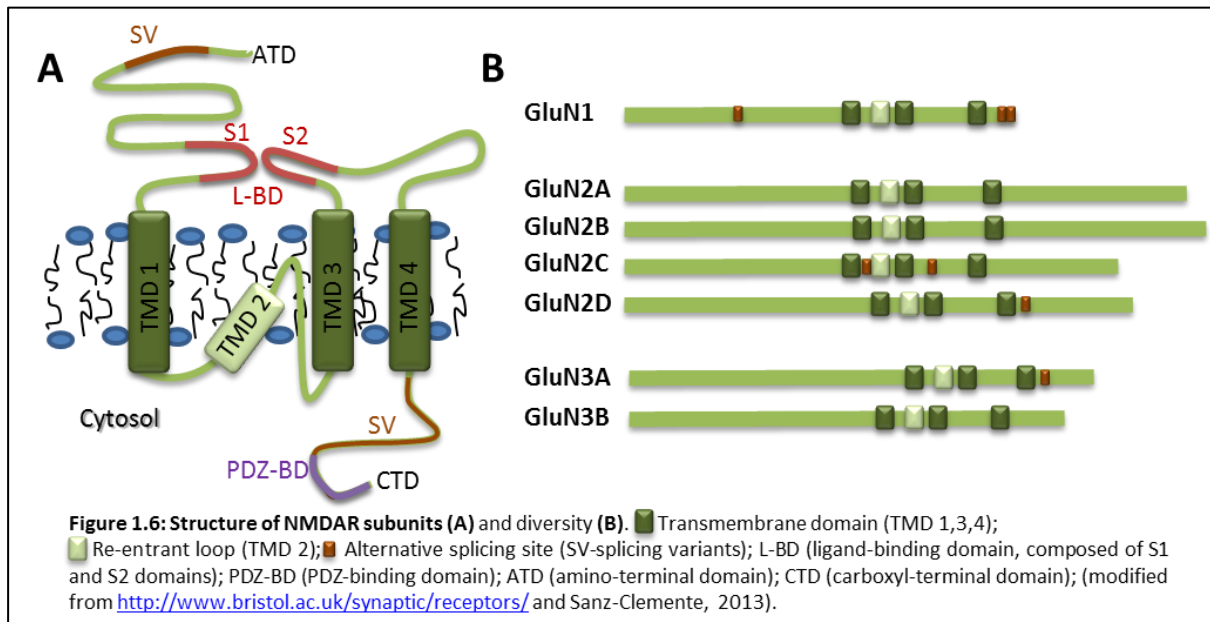
NMDAR subunits are encoded by three gene families, GluN1 (*Grin1*), GluN2 (*Grin2*), and GluN3 (*Grin3*). The GluN1 subunits are encoded by one gene; the GluN2 by four genes (designated A through D); and the GluN3 by two genes (A and B). Alternative splicing of the *Grin1* gene produces eight GluN1 isoforms (GluN1-1a/b, GluN1-2a/b, GluN1-3a/b, and GluN1-4a/b) (Anantharam et al., 1992; Hollmann et al., 1993). GluN1-a or -b indicates the absence or presence, respectively, of a splice cassette (exon 5) that encodes the amino-terminal domain of GluN1, while GluN1-1/2/3/4 indicate the splice variants of the carboxyl-terminal domain. The absence or presence of a splice exon 5 modifies the properties of NMDARs. For example, recombinant GluN1/GluN2 receptors lacking exon 5 are more sensitive to blockade by  $Zn^{2+}$  and protons and show stronger potentiation by polyamines through relief of proton inhibition (Hollmann et al., 1993; Traynelis et al., 1995; Traynelis et al., 1998).

## 3. NMDAR subunits

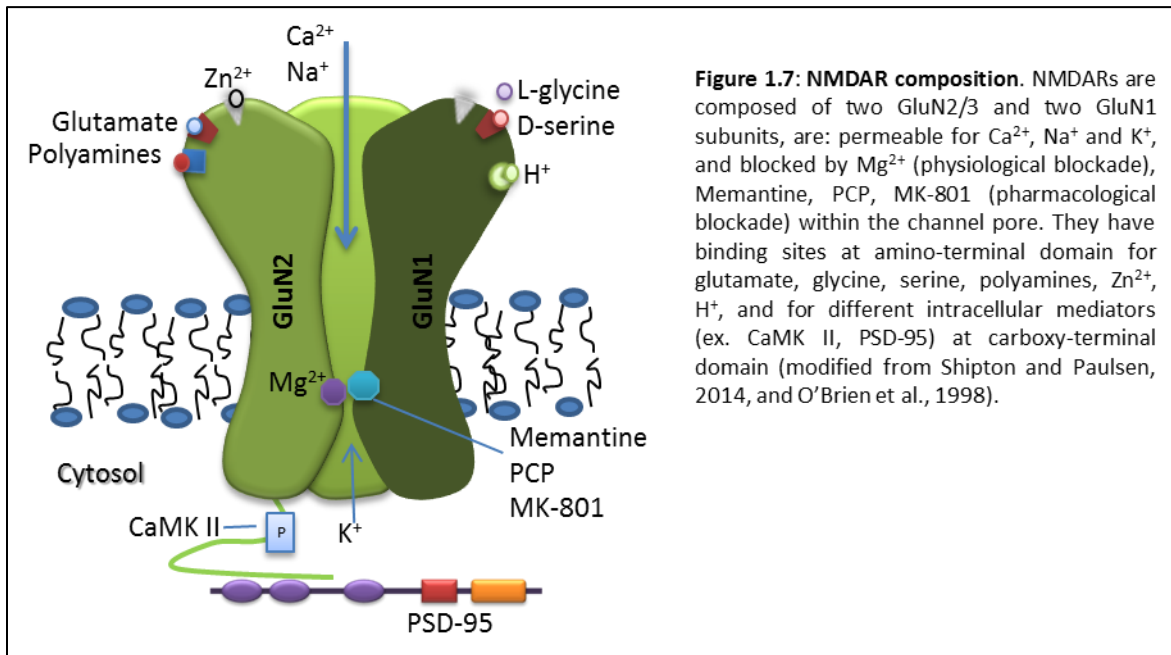
All NMDAR subunits have a common structure. They are composed of three transmembrane domains (TMD1, TMD3 and TMD4) and one re-entrant loop (TMD2). The long ATD region is extracellular, whereas **the carboxyl-terminal domain, whose length**



differs depending on subunits, is intracellular and interacts with multiple cytosolic proteins. Glutamate binds to GluN2 subunits in a binding place created by two regions: one present in the ATD (S1) and the other one present on the long extracellular loop between TMD3 and TMD4 (S2). The re-entrant TMD2 loop is part of the channel pore (Figure 1.6).



This TMD2 loop contains an asparagine residue that determines calcium permeability of the channel and mediates the magnesium blockade (Mayer and Armstrong, 2004). The extracellular ATD contains binding sites for allosteric modulators, such as  $Zn^{2+}$  and ifenprodil. The pore domain is permeable to ions and blocked by pore blockers, such as phenylcyclidine (PCP), MK801 and memantine and the CTD binds to different intracellular mediators (Shipton and Paulsen, 2014) (Figure 1.7). As an illustration, GluN2A and GluN2B subunit-containing NMDARs at the synapse are stabilized by their interaction with the PSD subfamily of membrane-associated guanylate kinases, which includes PSD-95, PSD-93, SAP102 and SAP97 (O'Brien et al., 1998). Noteworthy is the high affinity binding between the GluN2B CTD and the catalytic domain of  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) (Strack and Colbran, 1998), an interaction which plays a major regulatory role of synaptic plasticity and memory functions.



**Figure 1.7: NMDAR composition.** NMDARs are composed of two GluN2/3 and two GluN1 subunits, are permeable for Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup>, and blocked by Mg<sup>2+</sup> (physiological blockade), Memantine, PCP, MK-801 (pharmacological blockade) within the channel pore. They have binding sites at amino-terminal domain for glutamate, glycine, serine, polyamines, Zn<sup>2+</sup>, H<sup>+</sup>, and for different intracellular mediators (ex. CaMK II, PSD-95) at carboxy-terminal domain (modified from Shipton and Paulsen, 2014, and O'Brien et al., 1998).

Associate with different subunits of GluN2 (GluN2A-D) and GluN3 subunits (GluN3A and GluN3B), GluN1 forms several different NMDARs with distinct biophysical properties (Monyer et al., 1994) and specific patterns of expression during development and in the mature mammalian CNS (Dumas, 2005). NMDAR complexity is further enhanced through posttranslational modifications, such as phosphorylation, glycosylation, and ubiquitination, affecting cellular localization and function of the receptor (Dingledine et al., 1999).

#### 4. Expression and regulation

##### a) Cellular and subcellular expression of NMDAR subunits

NMDARs are widely distributed throughout the CNS and are principally expressed in neurons, though there are some indications that they could also be expressed on astrocytes (Lee et al., 2010b) and in endothelial cells (Legros et al., 2009). There are also some differences in the subcellular expression of the NMDAR subunits. GluN1 can be localized either in the plasma membrane within the complex formed by GluN2 and/or GluN3 subunits, or be retained in the endoplasmic reticulum (Huh and Wenthold, 1999). On the other hand, GluN2 subunits are mainly expressed at the plasma membrane of postsynaptic neuron, although there are some reports of presynaptic localization (Corlew et al., 2008). Expression of NMDAR subunits also varies depending on their synaptic vs non synaptic localization. GluN2A-containing NMDARs are predominantly expressed at synaptic sites whereas GluN2B-

containing NMDARs are present at both synaptic and non-synaptic sites in the adult CNS (Hardingham and Bading, 2010).

***b) Region-specific expression of NMDAR subunits***

The expression of individual NMDAR subunits is highly dependent on the brain area and developmental stage. In rodents, the GluN1-b variant of GluN1 (without the amino-terminal exon) is expressed mainly in the neonatal sensorimotor cortex, caudate nucleus, thalamus and CA3 sub-region of the hippocampus, while GluN1-a is expressed abundantly throughout the adult brain (Nakanishi et al., 1992; Laurie and Seeburg, 1994; Hoffmann et al., 2000). GluN2A levels are the highest in the adult hippocampus, cerebral cortex and thalamus. GluN2B subunit expression is strong in the adult cortex and hippocampus (Monyer et al., 1994). GluN3A and B mRNA are expressed highly in pons, midbrain, medulla, and the spinal cord.

***c) Expression of NMDAR subunits: from development to aging***

In rodents, the level of expression and composition of NMDAR subunits are dependent on the developmental stage. NMDAR subunit expression also varies as a function of the brain region considered (Flint et al., 1997a; Flint et al., 1997b; Liu et al., 2004b). During development, especially between the 7<sup>th</sup> and 14<sup>th</sup> postnatal day, the CNS becomes more sensitive to the toxic effects of glutamate. Also, long-duration NMDAR-mediated excitatory postsynaptic currents (EPSCs) become shorter and faster during the first postnatal week compared to the immediate afterbirth period (Lu et al., 2001). The reason for this enhanced vulnerability and fluctuations in the receptor kinetics has been suggested to be due to an increase in expression of specific NMDAR subunits (Miyamoto et al., 2001). Moreover, longer current duration and larger amplitude of EPSCs can be blocked by ifenprodil, a specific antagonist of GluN2B-containing NMDARs (Lu et al., 2001; Xing et al., 2006), and the level of expression of GluN2A subunit dramatically increases during the first week of postnatal development in rodents. It becomes a predominant subunit, although the overall levels of GluN2B expression do not change dramatically throughout development (Monyer et al., 1994; Hoffmann et al., 2000). Therefore, there is a change in the ratio of the two main NMDAR subunits and this particular GluN2B/GluN2A ratio is thought to be

responsible for the developmental-induced changing of NMDAR-mediated currents (Lu et al., 2001). GluN3A mRNA expression decreases sharply after the second postnatal weeks while GluN3B mRNA expression levels remain constant during postnatal development and into adulthood (Matsuda et al., 2002).

Further, in rodents, NMDARs are found to be more vulnerable to the aging process than other glutamate receptors. The GluN1 subunit has been observed to be particularly affected by aging. There are decreases in mRNA expression and protein levels of GluN1 subunit between mice of 3 and 30 months of age within the frontal and occipital cortices and the dentate gyrus of the hippocampus (Magnusson, 2000; Magnusson et al., 2002), but these reductions are not always consistent between studies involving aged C57BL/6 mice (Ontl et al., 2004; Zhao et al., 2009). Regarding GluN2A subunits, there is little or no effect of aging on the mRNA expression in the cortex or hippocampus of C57BL/6 mice (Magnusson, 2000), a resistance which contrasts with the decline in protein expression of the GluN2A subunit observed within the hippocampus of middle-aged and old C57BL/6 mice (Magnusson et al., 2002). The greatest age-related decline in NMDAR subunit expression in C57BL/6 mice concerns the GluN2B subunit. The mRNA and protein expressions of the GluN2B subunit decline significantly with age throughout the cerebral cortex and in the hippocampus (Magnusson et al., 2002; Ontl et al., 2004; Zhao et al., 2009). In the frontal cortex, the protein levels of the GluN2B subunit shows a greater decline with aging in the synaptic membrane fraction than in the whole homogenate (Zhao et al., 2009). This suggests that, in addition to the decline in mRNA expression of the GluN2B subunit, there may be an additional effect of aging on GluN2B subunit localization within the synaptic membranes of the frontal cortex.

## 5. Functional impact of subunit composition

NMDARs show diverse functional characteristics (sensitivity to  $Mg^{2+}$  and allosteric modulators, permeability for  $Ca^{2+}$ , de/activation kinetics and mean open time and maximal open probability of channel) in comparison to other glutamate receptors, which are also dependent on the subunits composition of NMDARs as well as the splice variants of GluN1 and/or GluN2 and GluN3 subunits.

**Receptor build-up of GluN2A and GluN2B subunits** compared to GluN2C and GluN2D subunits displays **higher sensitivity for blockade by extracellular  $Mg^{2+}$**  and higher permeability for  $Ca^{2+}$ . Those differences could be due to the difference in amino acid composition of the TMD2 region, which plays a critical role in  $Mg^{2+}$  blocking and  $Ca^{2+}$  permeability of the channel (Burnashev et al., 1992). Co-expression of GluN3B subunits with GluN1a and GluN2A subunits reduces the glutamate-induced  $Ca^{2+}$  influx (Matsuda et al., 2002).

Endogenous allosteric modulators, such as proton ( $H^+$ ) and zinc ( $Zn^{2+}$ ), have a different affinity for NMDARs subunits. Binding of proton, which leads to reduced open channel probability, is highest for GluN1-1a/GluN2B subunits, but is also dependent on both GluN1 and GluN2 subunits (Traynelis et al., 1995). In contrast, the **GluN1-1a/GluN2A subunit is the most sensitive to  $Zn^{2+}$**  (Dingledine et al., 1999).

Further, GluN1 subunits contain a steric barrier for glutamate at the agonist binding site due to the presence of tryptophan (Trp731) compared to GluN2 subunits which lack this steric barrier and possesses a valine (Val689) which causes the removal of hydrogen donor necessary for binding with carboxyl group of glutamate. In contrast to GluN2 subunits, this disparity leads to a higher sensitivity of GluN1 subunits to glycine but not to glutamate (Furukawa and Gouaux, 2003). **Affinity for glutamate also differs among the GluN2 subunits.** The highest sensitivity is shown by GluN2A subunits whereas the lowest concerns GluN2D (GluN2A<GluN2B/2C<GluN2D). In a same manner, **NMDARs composed of GluN1/GluN2A subunits have the fastest deactivation kinetic (40ms)**, and GluN1/GluN2D the slowest (2s). The observed deactivation time does not only depend on GluN2 subunits, but is also a function of the GluN1 splice variant. The variant with the amino acids encoded by exon 5 (GluN1b) accelerates the decay time course (Rumbaugh et al., 2000). **The maximal open probability of a channel is the highest for GluN1/GluN2A containing receptors**, intermediate for GluN1/GluN2B, and very low for GluN1/GluN2C and GluN1/GluN2D (Wyllie et al., 1998).

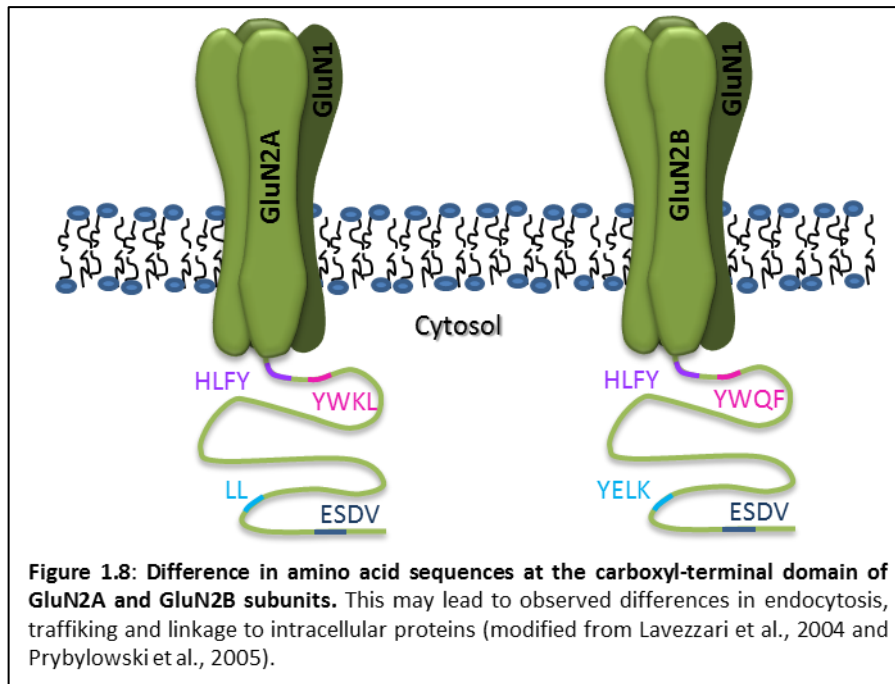
## 6. NMDAR trafficking

After protein synthesis, receptor subunits are assembled in the endoplasmic reticulum (ER) and next proceed to the Golgi apparatus and package in the Golgi complex by

means of vesicles, which in turn carry the NMDARs to the synapse. They are subsequently internalized and reinserted at extrasynaptic sites before being again anchored at the PSD area. At each step of this trafficking process, NMDARs need to be associated with specific partners that allow their maturation and adequate transport.

After synthesis in the ER, NMDARs are delivered to the synapse, a transport coordinated by the CTD of the GluN1 subunit (Prybylowski et al., 2005). Some of the GluN1 subunits, which can be detected in the intracellular compartment of the ER, are actually retained in the ER by an “ER retention signal” present in the alternatively spliced C1 cassette. The retention signal retains unassembled receptors in the ER. Moreover, binding of the GluN2 subunit masks the GluN1 retention signal, and promotes forward trafficking of the NMDARs to the postsynaptic membrane (Hall and Soderling, 1997; McIlhinney et al., 1998). The GluN1 subunit is produced in the ER in large excess relative to GluN2 subunits, ensuring that sufficient amounts of GluN1 subunits are available for newly synthesized GluN2 and/or GluN3 subunits (Chazot and Stephenson, 1997; Huh and Wenthold, 1999; Wenthold et al., 1999). Furthermore, the association of the receptor subunits with synaptic scaffolding proteins also participates to the trafficking of receptors from the ER to the postsynaptic cell surface (Standley et al., 2000; Scott et al., 2001). After being released from ER, NMDARs are further processed in the somatic Golgi apparatus and then distributed to the trans-Golgi network and endosomes, to finally reach the synaptic membrane (Horak et al., 2014).

At the synaptic membrane surface, receptors are internalized through the clathrin-coated pit pathway in the zone lateral to the synapse and PSD (Blanpied et al., 2002) called peri-synaptic. The domains necessary for the internalization are tyrosine-based internalization motifs at the distal CTD of GluN2 subunits and the clathrin adaptor protein 2 (AP2), which is thought to deliver NMDARs into clathrin-coated vesicles by lateral migration. The cytoplasmic CTD of GluN2A and GluN2B contain distinct internalization motifs that control their endocytosis and trafficking, which results in different internalization kinetics between subunits (Lavezzari et al., 2004; Groc et al., 2006). GluN2B is more mobile than GluN2A and it is subjected to lateral diffusion, endocytosis and recycling by an interaction between the YEKL motif of GluN2B and the AP2 complex. Slower internalization of GluN2A receptors is mediated by a dileucine motif (Lavezzari et al., 2004; Prybylowski et al., 2005) and AP2 (Figure 1.8).



NMDARs can take different routes following their internalization and incorporation in early/late endosomes; thus, GluN2A-containing NMDARs preferentially traffic to late endosome for degradation, while GluN2B-containing NMDARs tend to move to recycling endosomes from where they can return to the membrane before being addressed to the PSD area of the synapse (Lavezzari et al., 2004).

## 7. Functional impact of receptor location

### a) Diversity of NMDAR location

At the PSD, the receptors form a large macromolecular NMDAR complex together with scaffolding, adaptor and effector proteins that are involved in downstream signaling cascades and regulation of NMDAR function, membrane stability and trafficking. Perisynaptic NMDARs are located on the plasma membrane within 200–300 nm of the PSD (Petrulia et al., 2005; Zhang and Diamond, 2006), and are only activated by high glutamate concentrations in the synaptic cleft (Groc et al., 2009). Since receptors diffuse between synaptic and extrasynaptic sites (Tovar and Westbrook, 2002; Groc et al., 2004; Groc et al., 2006), the perisynaptic region may contain mobile receptors that are in transit to and from the PSD. Extrasynaptic NMDARs (extra-NMDARs) are localized at sites further from the PSD—on the spine neck, the dendritic shaft or soma (Tovar and Westbrook, 2002; Groc et al., 2006; Newpher and Ehlers, 2008). They can be activated after glutamate spillover from

synapse but many extrasynaptic NMDARs are adjacent to glia (Petrulia et al., 2010), suggesting that astrocytic release of glutamate may result in activation of these extrasynaptic NMDARs.

Initially, a controversial theory postulated dramatic localization differences between GluN2A- and GluN2B-containing NMDARs. While it is now clear that the partition is not absolute, the hypothesis that GluN2A is mainly synaptic and GluN2B is synaptic and extrasynaptic has received a fair amount of support over the years (Tovar and Westbrook, 2002; Liu et al., 2004b; Groc et al., 2006; Thomas et al., 2006; Harris and Pettit, 2008; Martel et al., 2009; Petrulia et al., 2010; Sanz-Clemente et al., 2013b).

Thus, extrasynaptic NMDARs can exert different functions compared to synaptically located NMDARs, a division of labor likely due to different subunit composition or association with different proteins (Rusakov and Kullmann, 1998).

#### ***b) Role(s) of scaffolding proteins in NMDAR location***

NMDARs are located at the synaptic and extrasynaptic compartments and are coupled to different proteins and intracellular signaling pathways (Hardingham and Bading, 2002; Ivanov et al., 2006). CTD of GluN2 subunits is responsible for the interactions with intracellular proteins, such as scaffolding proteins, especially proteins from the PSD-95 family (PSD-95, PSD-93, SAP-102, and SAP-97) (Kornau et al., 1995; Cousins et al., 2008). In particular, the two first PDZ domains of PSD-95 and the last four amino acids of GluN2 subunits (glutamate-serine-aspartate/glutamate-valine) are responsible for this association. Mutations (S1480A) or deletions of the last amino acids of the NMDAR subunits lead to abolished or reduced interaction with scaffolding proteins (Prybylowski et al., 2002; Prybylowski et al., 2005).

Interaction of SAP-102 and PSD-95 with NMDARs has been shown to traffic the receptors to synapses from endoplasmic reticulum (Sans et al., 2003), and to regulate cell surface expression via inhibition of internalization (Lavezzari et al., 2004). Phosphorylation of Ser1480 disrupts the interaction of GluN2B with the PSD-95 and SAP102 proteins, and decreases surface GluN2B expression in neurons (Chung et al., 2004). Disruption of GluN2A and scaffolding protein association, by biomimetic divalent competing ligand, lead to



increased surface diffusion of GluN2A-containing NMDARs, highlighting the anchoring role of PDZ scaffolds (Bard et al., 2010).

**c) *Functional consequences of extrasynaptic NMDAR activation***

Activation and recruitment of NMDARs are necessary for the modulation of synaptic plasticity, including LTP and LTD mechanisms, which are thought to underlie learning and memory processes. Contrasting with this beneficial effect, activation of these receptors could also trigger excitotoxicity and lead to neuronal death. These two opposite outcomes were initially considered to be due to different degree of  $\text{Ca}^{2+}$  entry through NMDARs. Moderate activation of NMDARs was considered to have positive effect on cell processes and survival, while over-activation of NMDARs could “overload” the cell with  $\text{Ca}^{2+}$  and trigger deleterious effects. While this mechanism remains valid, additional studies have revealed that the so-called “NMDAR paradox” could be more precisely explained by taking into consideration the location of these receptors. Accordingly, synaptically-located NMDARs (syn-NMDARs) are considered to predominantly mediate beneficial effects for the cell, while NMDARs located extrasynaptically (extra-NMDARs) are coupled to cell death pathways which, when triggered, lead to neuronal degeneration and eventually neuronal death (Hardingham and Bading, 2002; Ivanov et al., 2006; Leveille et al., 2008).

The mechanisms underlying the neuroprotective effect of syn-NMDARs include induction of “survival” genes, suppression of “death” genes and mitochondrial protection. Flux of calcium from the extracellular compartment, after syn-NMDAR activation, along with the release of internal calcium stores, leads to a significant increase of this ion inside the cell.  $\text{Ca}^{2+}$  migrates to the nucleus and there, activates various genes. In the nucleus, calcium binds to the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase IV (CaMK IV) and cyclic-AMP response element binding protein (CREB) (Hardingham and Bading, 2002; Zhang et al., 2011a). The transcription factor CREB, after activation by  $\text{Ca}^{2+}$ , increases the expression of the gene encoding neurotrophin brain-derived neurotrophic factor (BDNF) (Favaron et al., 1993; Jiang et al., 2005), or others genes that may provide the neuroprotection of mitochondria or activate anti-apoptotic pathways (Zhang et al., 2007a). Calcium entry through syn-NMDARs, but not through extra-NMDARs, may also inhibit the expression of pro-death genes (e.g.

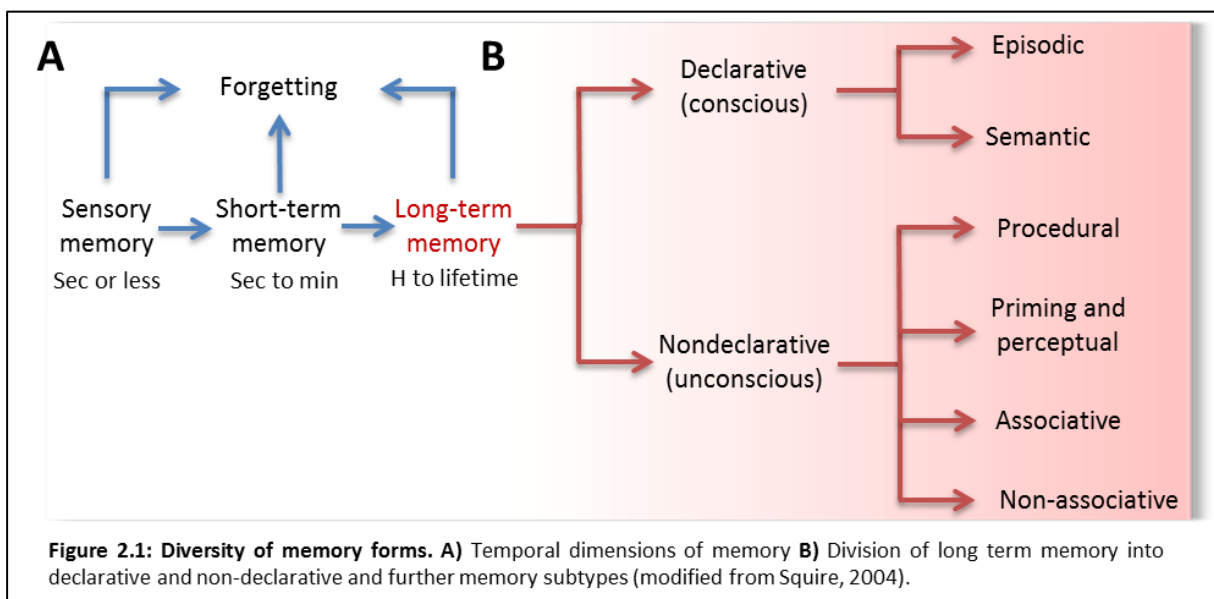
*puma*, *bim*, *fasl* and other) by suppressing the activity and expression of pro-death transcriptional factors (e.g. Forkhead box O-FOXO) (Al-Mubarak et al., 2009).

In contrast, extra-NMDARs  $\text{Ca}^{2+}$  influx leads to CREB inactivation (Hardingham and Bading, 2002; Zhang et al., 2011a; Kaufman et al., 2012; Chen et al., 2014), FOXO activation or the inactivation of the extracellular signal regulated protein kinase 1/2 (ERK1/2). In addition, activation of extra-NMDARs also recruits pathways not involved in syn-NMDAR signaling. For example, Syn-NMDAR activation does not affect calpain activity, whereas extra-NMDAR stimulation induces calpain-mediated cleavage of striatal enriched tyrosine phosphatase (STEP) into an inactive form (Xu et al., 2009). This inactive form of STEP is unable to dephosphorylate its substrates, including the stress-activated protein kinase, p38, and the Src kinase family member, Fyn, leading to an overactivation of these substrates following STEP cleavage. Activation of p38 is closely linked with cell death (Kawasaki et al., 1997); however, while recently recognized, the consequences of increased Fyn activity are less clear. One consequence of increased Fyn activity is an increase in the surface expression of Glu2B (Xu et al., 2009). Fyn phosphorylates the Tyr1472 residue of the GluN2B subunit, leading to exocytosis of NMDAR complexes to neuronal surfaces (Dunah et al., 2004). When extra-NMDAR stimulation inactivates STEP, STEP can no longer deactivate Fyn, leading to increased Fyn activity and exocytosis of Glu2B receptors (Hallett et al., 2006). In addition, STEP dephosphorylates the Tyr1472 residue of GluN2B, promoting internalization (Snyder et al., 2005). Thus, inactivating STEP leads to decreased endocytosis and increased exocytosis of GluN2B receptors (Hallett et al., 2006). This increase in GluN2B-containing receptors may then lead to an increase in extra-NMDAR signaling, thereby creating a self-perpetuating, feed-forward loop of excitotoxicity.

## II. NMDARs IN LEARNING AND MEMORY

## A. Types of memories

Learning and memory processes are fundamental to human life allowing us to acquire new knowledge that can be used to plan into the future and respond adaptively to unfamiliar situations. In humans, memory can be divided into short-term and long-term memory (Baddeley, 2001) (Figure 2.1). **Short-term memory** lasts on the scale of seconds to a few minutes. These memories are readily lost and are very sensitive to disruption. **Working memory**, often called 'online memory' is a subtype of short-term memory that allows us to temporarily store and manipulate information. It requires attention, active repetition and it is limited in its storage capacity (Baddeley, 1981). **Long-term or remote memories**, on the other hand, are long-lasting, with virtually unlimited storage capacity. **They undergo a process of consolidation before being available for reliable retrieval.**



Long-term memory can be grossly divided into two types: declarative and nondeclarative memory. **Declarative memory**, also known as explicit memory is the memory for facts (semantic memory) and events (episodic memory) (Tulving and Schacter, 1990). It enables conscious recollection, depends on medial temporal lobe structures and is very sensitive to amnesia. Declarative memory underlies our representation of the outside world, it allows us to encode the relationship between multiple items and events, it is flexible and the stored material can guide our performance in a variety of situations (Squire, 1992). **Non-**

**declarative, or implicit, memory** on the other hand is acquired through experience and can be readily accessed when the circumstances require it. It is expressed through performance and does not require a conscious recollection of the past. The recall of these memories occurs through the reactivation of the systems through which the learning had occurred originally. Non-declarative memory can be further subdivided into i) procedural memory - the memory for skills, habits, ii) priming and perceptual learning iii) non-associative learning - such as sensitisation and habituation, as well as iv) associative learning such as simple forms of classical conditioning (Squire, 1992, 2004). **Despite this categorization, it is important to keep in mind that in most life situations multiple forms of memory are activated in parallel.**

## **B. Consolidation**

Engrams refer to the physical entity in the brain that stores information over time and later enables memories to be expressed. There are many determinants of the persistence of these engrams also referred to as memory traces). A common and widely accepted thread is that memory encoding and initial storage are followed by a consolidation process that, when triggered, enables traces to mature and become stabilized over time. Thus **consolidation can be defined as a process that transforms new and initially labile memories encoded in the awake state into more stable representations that become integrated into the network of pre-existing long-term memories.**

Neurobiologists distinguish between two types of memory consolidation — one fast, one slow — and their different kinetic properties reflect qualitatively distinct underlying processes. The faster process, called “**synaptic consolidation**” concerns the more immediate determinants of synaptic strength and persistence that are triggered within individual neurons in the minutes and hours after memory encoding. These changes are necessary for the initial stabilization of memories and typically occur in the same neuronal circuits that participated to encoding. Synaptic consolidation primarily involves morphological changes within localized neuronal circuits, such as the restructuring of existing synaptic connections or the growth of novel ones. These changes are triggered by a cascade of different metabolic events which include activation of central receptors, release of different neurotransmitters and second messengers, activation of transcription factors and associated target genes, and

ultimately the synthesis of a plethora of proteins directly responsible for the observed structural changes.

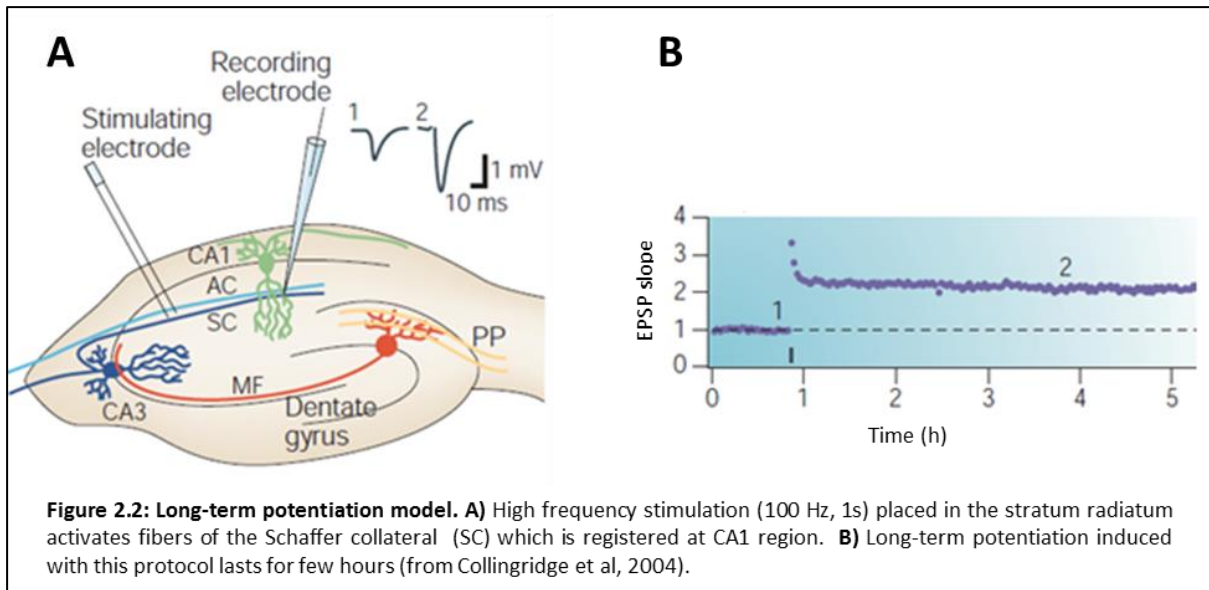
The slower process, called “systemic consolidation” can last for weeks to months, or even up to several decades, depending on the species. Consolidation in this case refers to a gradual process of reorganization and a differential involvement of the brain regions that support memory processing. For instance, the brain regions that are crucial for processing recently acquired information are not necessarily identical to those that are important for later memory recall. The mechanisms underlying this reorganization are largely unknown.

### 1. **Synaptic consolidation**

Ramon Y Cajal originally hypothesized that information storage relied on changes in strength of synaptic connections between neurons that were active (Cajal, 1913). Hebb supported this hypothesis and proposed that if two neurons are active at the same time, the synaptic efficiency of the appropriate synapse would be strengthened (Hebb, 1949). It is now widely accepted that **memory formation is dependent on changes in synaptic efficacy that permit strengthening of associations between neurons**. An enormous effort has been put into understanding the mechanisms by which strengthening of synaptic connections can be achieved. This effort has led to the proposal of two key models of neuronal plasticity triggered during synaptic consolidation: **long-term potentiation and long-term depression**.

#### *a) Long-term potentiation*

Long-term potentiation (LTP) is an artificial model that is thought to mimic the physiological bases of learning and memory processes. It was initially demonstrated in 1973 by Bliss and colleagues. They showed that high frequency stimulation of the perforant path in the hippocampus resulted in a persistent increase in the efficiency of synaptic transmission in the dentate gyrus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) (Figure 2.2).

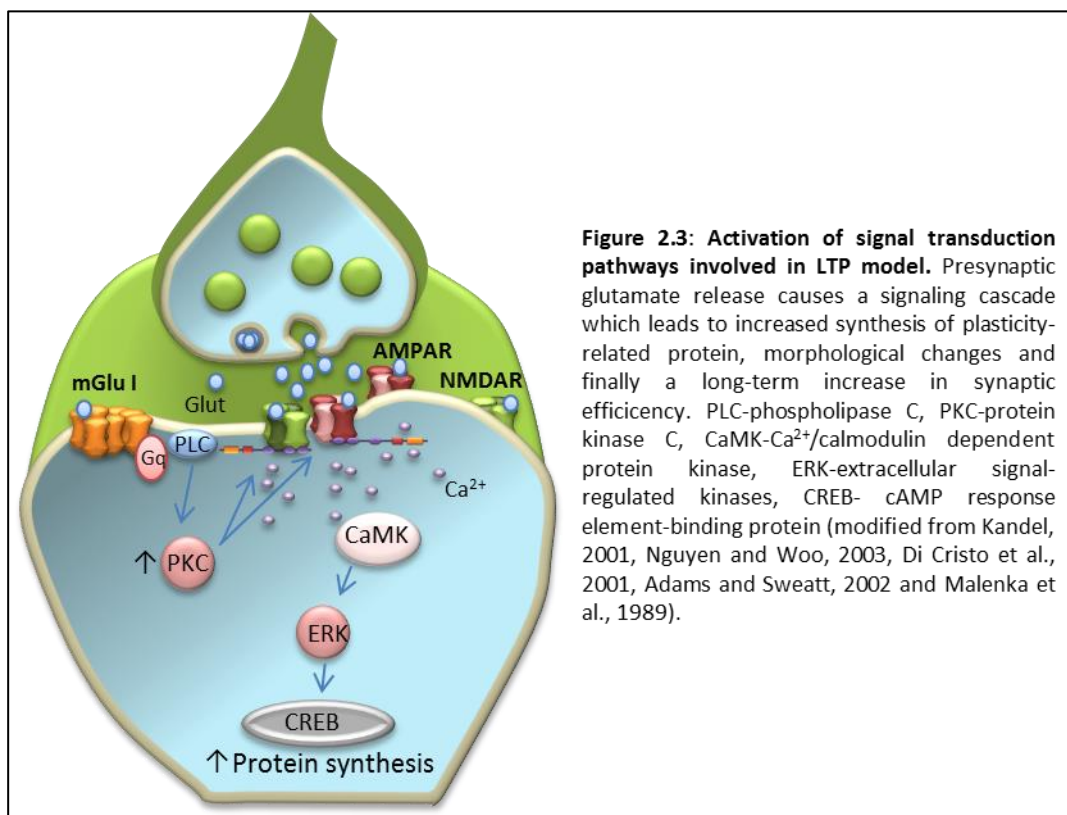


LTP can persist for hours *in vitro* and several days when induced by electrical stimulation *in vivo* (Douglas and Goddard, 1975) and its modulation causes changes in the learning ability of experimental animals. Indeed, various studies have shown that inhibition of LTP affects memory performance in animals (Morris et al., 1986; Staubli et al., 1989). It is well known now that this process depends on a cascade of events that is initiated by synaptic activation, which leads to the recruitment of NMDARs, second messenger systems, activation of transcription factors and, ultimately, synthesis of new proteins required for persisting structural and functional changes within neuronal networks.

The onset of LTP involves post-synaptic  $\text{Ca}^{2+}$  entry. Indeed  $\text{Ca}^{2+}$  chelators or reduction of the extracellular  $\text{Ca}^{2+}$  concentration blocks the triggering of LTP (Lynch et al., 1983). NMDARs are the main actor responsible for the  $\text{Ca}^{2+}$  entry involved in LTP (Collingridge et al., 1983; Nicoll and Malenka, 1999) even if the activation of voltage-gated calcium channels (Morgan and Teyler, 1999) or metabotropic receptors mGluR1 and mGluR5 (Bashir et al., 1993) may also induce LTP, which is referred to as NMDAR-independent LTP.

After glutamate release, if the postsynaptic cell is sufficiently depolarized, NMDAR blockade by magnesium can be removed allowing an influx of  $\text{Ca}^{2+}$ . Intracellular  $\text{Ca}^{2+}$  induces the activation of intracellular transduction pathways including CaMKII (Malenka et al., 1989), protein kinase A (PKA) (Roberson and Sweatt, 1996; Impey et al., 1998) and protein kinase C (PKC) (Lynch, 2004; Schmitt et al., 2005). The activation of these protein kinases triggers phosphorylation of the AMPARs and alter the intrinsic properties of these ligand-gated ion

channels (increased activity or increased cell surface insertion) (Malenka and Bear, 2004). Moreover, CaMKII, PKA and PKC may affect ERK1/2 and CRTC1 (Di Cristo et al., 2001; Adams and Sweatt, 2002). The activation of ERK1/2 (Kelleher et al., 2004) and consequently the recruitment of the Transducer of regulated CREB activity 1 (TORC1) (Kovacs et al., 2007) increase CREB's transcriptional efficacy of genes encoding neurotrophins (Impey et al., 1996; Lu et al., 2014). In this late phase of LTP, activation of the signal transduction pathways mentioned above also causes an increase in plasticity-regulated proteins associated with morphological changes and increased synaptic efficiency (Kandel, 2001; Nguyen and Woo, 2003) (Figure 2.3).



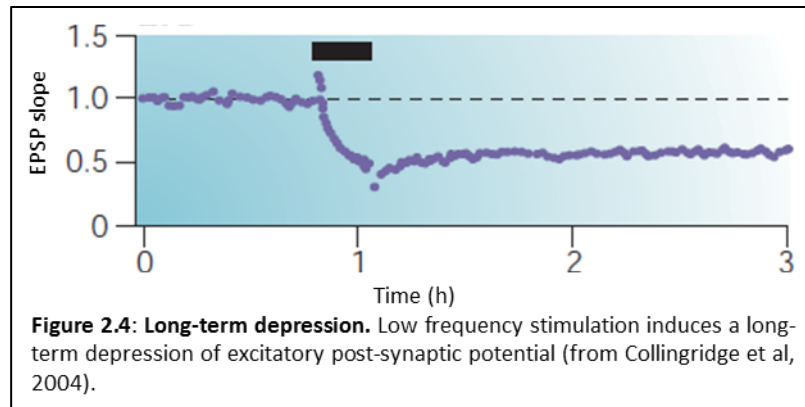
**Figure 2.3: Activation of signal transduction pathways involved in LTP model.** Presynaptic glutamate release causes a signaling cascade which leads to increased synthesis of plasticity-related protein, morphological changes and finally a long-term increase in synaptic efficiency. PLC-phospholipase C, PKC-protein kinase C, CaMK- $Ca^{2+}$ /calmodulin dependent protein kinase, ERK-extracellular signal-regulated kinases, CREB- cAMP response element-binding protein (modified from Kandel, 2001, Nguyen and Woo, 2003, Di Cristo et al., 2001, Adams and Sweatt, 2002 and Malenka et al., 1989).

### ***b) Long-term depression***

Long-term depression (LTD) can be considered as the opposing process to LTP. It can be induced by low frequency stimulation of the presynaptic neuron (1-5 Hz) (Dudek and Bear, 1992). Just as for LTP, some forms of LTD are dependent upon NMDARs. Low concentration of  $Ca^{2+}$  influx through these receptors activates calcium-responsive phosphatases such as type 1, , types 2A and 2B phosphatases (PP1, PP2A, PP2B also called calcineurin) (Mulkey et al., 1994). Consequently, these phosphatases dephosphorylate



AMPA receptors (Morishita et al., 2005) and kinases responsible for phosphorylation of AMPARs (Blitzer et al., 1998), dephosphorylate AMPARs and can induce a decrease of the number of these receptors at the postsynaptic membrane (Beattie et al., 2000) (Figure 2.4).



## 2. Systemic consolidation

Systemic, also called systems-level, memory consolidation is a highly dynamic process that involves large scale reorganization at the level of entire interconnected brain systems. Consolidation, in this case, refers to a gradual and slow process of reorganization of the different brain regions that support memory stabilization over time (Dudai, 2004; Squire and Bayley, 2007; Wang and Morris, 2010). According to the so-called **standard theory of systems-level memory consolidation**, the hippocampus is believed to integrate information transmitted from distributed cortical networks that support the various features of a whole experience (Squire and Alvarez, 1995). Upon encoding, the hippocampus rapidly fuses these different features into a coherent memory trace.

Consolidation of this new memory trace at the cortical level then **occurs slowly via repeated and coordinated reactivation of hippocampal-cortical networks during sleep** in order to progressively increase the strength and stability of cortical-cortical connections between tagged hippocampal and cortical neurons which represent the original experience (Lesburgueres et al., 2011). Over days to weeks, as memories mature, the role of the hippocampus would gradually diminish, presumably leaving cortical areas to become capable of sustaining permanent memories and mediating their retrieval independently (Squire and Alvarez, 1995; Frankland and Bontempi, 2005). The time course of this process, which can take weeks, months or even years, is consistent with the fact that lesions or pharmacological inactivation of the prefrontal or orbitofrontal cortices in rats and the

anterior cingulate cortex in mice disrupt recall of remote, but not recent, memories, whereas inactivation of the hippocampus impairs recent, but not remote memories (Bontempi et al., 1999; Wiltgen et al., 2004; Ding et al., 2008; Lesburgueres et al., 2011).

It is important to note that not all the experimental evidence supports the standard model of memory consolidation. This has led some researchers to propose an alternative view, known as the multiple trace theory, in which the hippocampus is necessary for memory recall as long as memories are viable (Nadel and Moscovitch, 1997). According to this theory, each memory trace, which corresponds to a specific conscious experience, consists of a cohesive hippocampal–cortical ensemble. Each time that a particular event is recalled, this event is recoded in the form of multiple related memory traces dispersed over larger areas of the hippocampus. Consequently, the relative sparing of remote memories in amnesic patients is a function of the extent of hippocampal damage, with limited damage producing temporally graded retrograde amnesia and extensive lesions resulting in a flat gradient for retrograde amnesia. Another interesting feature of this model is that the form of memory being processed is thought to determine the time course of hippocampal engagement during consolidation. Whereas the standard model favors a disengagement of the hippocampus regardless of the type of declarative memory (semantic or episodic) being processed, the multiple trace theory predicts that complete hippocampal lesions will produce temporally graded retrograde amnesia only for semantic (general knowledge of facts) but not episodic (richly detailed recollection of specific events) memory. Some case studies, for instance, patient E.P., who has extensive bilateral lesions of the medial temporal lobe but excellent autobiographical memories from his youth, do not support this prediction. Furthermore, there are several examples of temporal gradients in animals even after complete hippocampal lesions. A central issue, still unresolved, is whether remote episodic memories following extensive hippocampal lesions are as detailed and vivid as those in control subjects (Frankland and Bontempi, 2005).

### 3. **Role of reactivation or replay during the sleep**

#### *a) Slow wave ripples*

The hippocampus encodes information during active wakefulness. These memory traces are thought to be subsequently reactivated within hippocampal-cortical networks

during online states of conscious recollection (or quiet wakefulness) or during offline periods such as sleep, facilitating their progressive embedding and long-term storage within cortical networks via the triggering of weight (LTP/LTD-dependent mechanisms) and wiring (creation of novel connections and/or suppression of existing ones) plasticity phenomena. This memory reactivation process, which is the core mechanism in most contemporary models of memory consolidation, is supported, at least in part, by hippocampal field potential oscillations. Indeed, during slow wave sleep (SWS) and periods of quiet wakefulness, the hippocampus displays large irregular activity, characterized by recurring occurrences of brief (80 ms) high-frequency bursts (100–250 Hz) that appear in the local field potential (LFP). These burst of activity are called **sharp-waves ripples** (SWRs) (Buzsaki et al., 1992). Since SWRs provide the strong synaptic bombardment necessary for the induction of long-term synaptic changes, SWRs were proposed as a candidate mechanism for offline consolidation (Buzsaki, 1989; Girardeau and Zugaro, 2011). More recently, several studies have revealed a **correlation between SWRs and memory**. Ripple occurrence rates increase during the first hour following a training session in an odor-reward association task (Eschenko et al., 2008). A similar increase was observed in rats learning a radial maze task, concomitant with a significant improvement in performance (Ramadan et al., 2009). Taken together, these studies suggest that SWRs may be modulated by learning-related regulatory processes. However, the first direct evidence for a **causal role of SWRs in memory consolidation** was provided in several studies where ripples were selectively suppressed, a manipulation which resulted in impaired consolidation (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010). The suppression just after the training of automatically-detected ripples by timed electrical stimulation can result in performance impairment in spatial memory tasks (Girardeau et al., 2014). Although these studies clearly establish a causal link between SWRs offline memory consolidation, they leave one unanswered key question. Indeed, **it is not clear whether the observed deficit was induced by the absence of ripples or the disruption of memory reactivations**. We attempted, at least, partially, to address this question by studying the functional contributions of hippocampal SWRs in Alzheimer’s disease (Results Part C).

### ***b) Spindles***

During slow wave sleep, neocortical field potentials oscillate at 0.1–4 Hz. This slow oscillation reflects the synchronous alternation of widely distributed populations of

neocortical neurons between depolarized (or up-state) and hyperpolarized (or down-state) states, corresponding to periods of high activity and almost complete silence (Steriade, 2003a). Experimental induction of slow oscillations by transcranial magnetic stimulation enhances memory performance, indicating that neocortical slow oscillations are also critically involved in memory consolidation (Marshall et al., 2006). Slow oscillations are accompanied by faster (7–15 Hz) transient oscillations, termed thalamo-cortical spindles (Steriade, 2003b), which have also been linked to learning and memory both in humans and animals (Gais et al., 2002; Eschenko et al., 2006). Slow oscillations and spindles are correlated with hippocampal large irregular activity. Indeed, hippocampal SWRs are more likely to occur at the transition between down and up-states (Sirota et al., 2003; Battaglia et al., 2004; Isomura et al., 2006; Molle et al., 2006) and coincide with neocortical spindles (Siapas and Wilson, 1998; Sirota et al., 2003; Molle et al., 2009; Clemens et al., 2011). Such a coordinated replay of experience-dependent activity could thus represent one important mechanism that drives not only stabilization and refinement of memory traces in the cortex but perhaps also the updating of pre-existing cortical memories (Siapas and Wilson, 1998; Ribeiro and Nicolelis, 2004).

**Remarks:** These studies support the idea that consolidation of memory traces at the cortical level may require the SWR-related reactivation of neuronal ensemble activity within hippocampal networks during offline periods including slow wave sleep. However, experimental proof of a causal link between SWRs, slow oscillations and spindles and hippocampal-neocortical interactions leading to remote memory storage is still missing.

## C. Role of NMDARs in learning and memory

### 1. NMDARs and memory formation

Since the seminal discovery by the Morris's laboratory that the hippocampal infusion of AP5, a selective competitive antagonist of NMDARs, impairs the development of LTP and the learning abilities in hippocampal-dependent spatial tasks (Morris et al., 1986; Abraham and Mason, 1988), several studies have characterized the role of NMDAR function in memory processes. To selectively inactivate global NMDARs or specific subunit of NMDARs, several strategies have been used ranging from classical pharmacological blockade to the use of genetically-engineered mice.

According to the studies summarized in annex 1, NMDARs play a crucial role in encoding (Morris et al., 1986), cellular tagging (Lesburgueres et al., 2011), replay (Girardeau, 2014), extinction (Dalton et al., 2012) and recall (Day et al., 2003; Corcoran et al., 2011a) and even forgetting (Shinohara and Hata, 2014). However, there are still some unsolved issues as to the role of NMDAR composition and location in memory processes.

	GluN1/ GluN2A	GluN1/ GluN2B	
Development	--	++	Neuronal expression
Adulthood	+++	++	
Low neuronal activity	↓	↑	
High neuronal activity	↑	↓	
LTP phase engagement	late	early	
Deactivation speed	118 ms	600 ms	Receptor kinetics
EC <sub>50</sub> glutamate	1.7 μM	0.8 μM	
EC <sub>50</sub> glycine	2.1 μM	0.3 μM	
CaMK II	-	+	Intracellular interaction
Casein kinase2	-	+	
ERK signaling	-	+	
Spine stability	+	-	Synaptic plasticity
Structural plasticity	-	+	
Functional plasticity	-	+	

Figure 2.5: Differences in expression, regulation and properties of GluN2A- and GluN2B-containing NMDARs.

Building upon the NMDAR involvement in memory processes and the distinct biophysical properties of GluN2A and GluN2B subunits (Figure 2.5), it seems that GluN2B subunits, by facilitating NMDAR-mediated synaptic plasticity, may be predominantly required in the early stages of memory consolidation to encode new information at the synaptic level or when the brain needs to re-encode new memory during reconsolidation as observed by the Nader's group (Ben Mamou et al., 2006) or even during extinction/forgetting processes (Corcoran et al., 2013). **GluN2B could be required to model or destabilize the synapse and/or the neuronal network, in order to encode, re-encode or erase information.** In contrast, **GluN2A subunits could be required to stabilize the information or for recall, and**

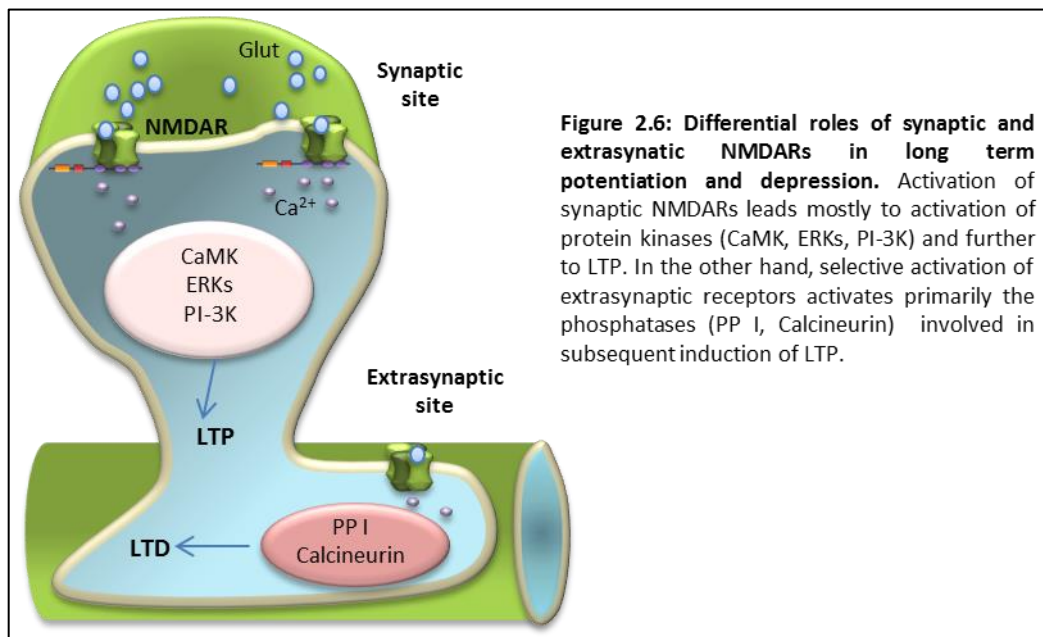
**could protect synapse and/or neuronal networks against remodelling and subsequent forgetting.** Thus, a shift between GluN2B to GluN2A, as observed by several groups *in vivo* (Quinlan et al., 2004) but also *in vitro* during LTP (Bellone and Nicoll, 2007; Yashiro and Philpot, 2008), may drive the progressive embedding and stabilization of long-lasting memories during the course of the synaptic and systems-level memory consolidation processes.

Thus, following initial encoding by neurons, which has been allocated (i.e. “tagged”), for the task, recurrent reactivations of the hippocampal-cortical network via hippocampal ripples and/or cortical spindles might progressively increase the synaptic GluN2A/GluN2B ratio. Further, this might lead to the shortening of NMDAR-mediated postsynaptic currents, which may constrain NMDAR-dependent synaptic changes and therefore, ensure the stabilisation and storage of memory traces at the cortical level, protecting them from interference and forgetting. The work to validate this hypothesis, currently only based on the literature and properties of NMDAR subunits is ongoing in our team.

## **2. Role of NMDAR location in synaptic plasticity and memory formation**

It has been shown that the selective activation of synaptic NMDARs is neuroprotective in various models of apoptosis (Papadia et al., 2005; Soriano et al., 2006), involving the activation of CaMK proteins (Lee et al., 2005b), ERK1/2 (Ivanov et al., 2006) and also the phosphatidylinositol 3-kinase (PI-3K) (Papadia et al., 2005). The activation of synaptic NMDARs also phosphorylates the CREB transcription factor and the subsequent expression of the growth factor BDNF (Hardingham et al., 2002). All of these factors have been described as actors for LTP and memory formation, confirming that the synaptic NMDARs are major regulators of synaptic plasticity and learning. While the role of extra-NMDARs is still unknown, indirect evidence however suggests that recruitment of extra-NMDARs could block the synaptic NMDARs and slowdown synaptic activity and consequently alter memory formation. First, stimulation of extra-NMDARs blocks the intracellular signaling cascade induced by syn-NMDARs, such as ERK1/2 and CREB (Hardingham et al., 2002; Ivanov et al., 2006), by increasing phosphatase activities (Sheng and Kim, 2002). Almost all these signaling pathways have been involved in synaptic plasticity

and/or memory formation (Giese and Mizuno, 2013). Second, studies have considered LTD to be induced via GluN2B-containing NMDARs located extrasynaptically (Massey et al., 2004; Li et al., 2009; Potier et al., 2010). Third, there are converging evidence showing that some electrophysiological disturbances in aged rats could be triggered by a facilitated activation of extra-NMDAR (Potier et al., 2010), leading to age-related memory deficits. Fourth, increasing of extracellular glutamate, via cannabinoid receptor 1, activates extrasynaptic GluN2B-containing NMDARs and triggers AMPAR internalization. These events ultimately induce LTD, altering the function of hippocampal network that likely become unable to process spatial working memory (Han et al., 2012) (Figure 2.6). Finally, there is an increasing number of publications suggesting a role of extra-NMDARs in CNS disorders (Parsons and Raymond, 2014), and more especially in the cognitive disorders associated with AD (see below paragraph: Role of NMDARs in AD).



## Conclusion

NMDARs are key actors for synaptic plasticity and memory formation. NMDAR subunit composition (GluN2B/GluN2A ratio) and NMDAR location are two main determinants controlling the fate of neurons (survival versus death) and the organization of recent and remote memories (stabilization versus forgetting of the memory trace). Accordingly, pathological alterations in the expression of GluN2B and GluN2A subunits,

GluN2B/GluN2A ratio or in the syn-NMDARs/extra-NMDARs ratio can impact synaptic plasticity and learning and memory processes.



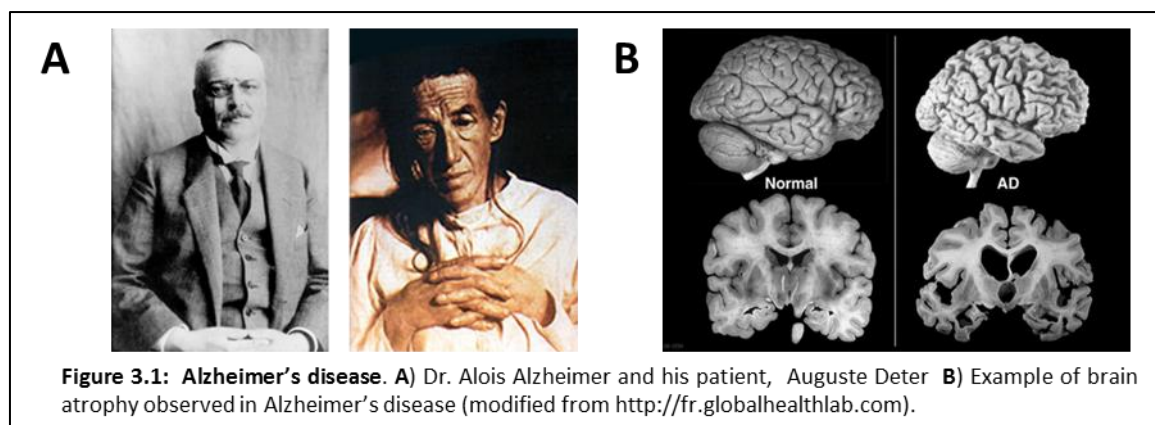
### III. ALZHEIMER'S DISEASE

## A. Alzheimer's disease: a small story from the past, a big challenge for the future

Alzheimer's disease (AD), the most frequent form of dementia, is a progressive, degenerative brain disease characterized by impairment of cognitive, behavioral and functional abilities that principally affects the elderly. The disorder slowly progresses to a loss of functional abilities and finally to complete dependency. Given the disproportionate rise in the number of elderly people and the social, economic and health care impact, AD is increasingly being recognized as one of today's major healthcare **challenges**.

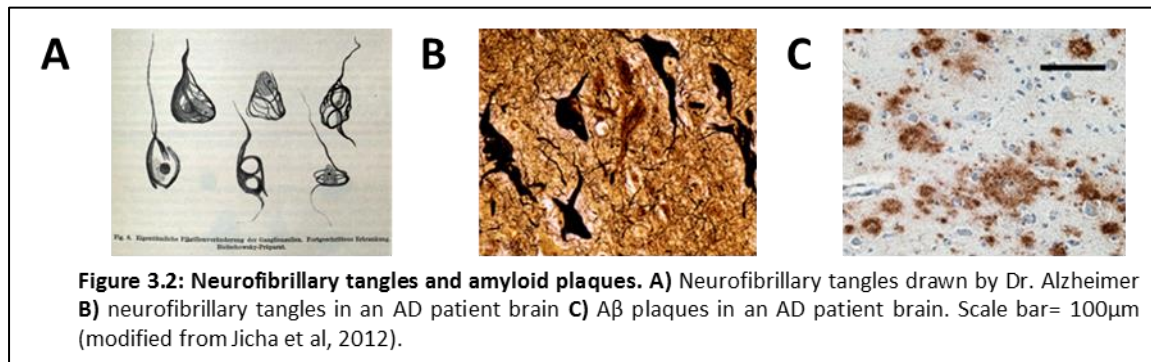
### 1. Dr. Alois Alzheimer and Alzheimer's disease: a short story from the past

Alzheimer's disease as the "presenile dementia" was described for the first time in 1906 by Dr. Alois Alzheimer (1864-1915), a German neuropathologist and psychiatrist, at the Tübingen meeting of the Southwest German Psychiatrists. From 1901, Dr. Alois Alzheimer treated a 51-year-old woman, Auguste Deter, who had progressive memory loss, reduced comprehension, aphasia, disorientation, paranoia, hallucinations, delusions and pronounced psychosocial impairment. This patient had become demented at a relatively young age. After she died, 5 years after hospitalization, the autopsy revealed the atrophy of cerebral cortex and widened sulci (Figure 3.1).



A new silver tissue-staining method and greatly improved microscopes revealed numerous small miliary foci –globs of sticky proteins in the spaces between neuron cells and conglomerated fibers within cells – in today's terminology senile plaques and neurofibrillary tangles (Alzheimer et al., 1995; Shampo et al., 2013) (Figure 3.2). In the following years, and

in collaboration with an Italian psychiatrist and his disciple, Gaetano Perusini, Dr. Alzheimer identified several other cases of “presenile dementia”. These findings, published in 1909, again confirmed that senile plaques and neurofibrillary tangles were the likely pathological substrates of dementia.



Although, the description of plaques and tangles was not a novel finding in 1906, they were not tagged as markers of disease. The presence of plaques was firstly reported by Paul Blocq and Georges Marinesco in 1882, in a patient suffering of some form of epilepsy. Plaques were also reported by Solomon Carter Fuller in a patient suffering from senile dementia 5 months before Dr. Alzheimer’s presentation. Dr. Alzheimer only wanted to draw attention to the fact that senile plaques could occur at younger ages than previously thought. He had no intention of proposing a new disease entity.

Dr. Emil Kraepelin, also German psychiatrist and head of Dr. Alzheimer, believed that most mental disorders had organic origin, as opposed to his rival, Sigmund Freud, who stated that most mental illnesses were *psychoses of the mind*. He believed that with good methods for examining the brain tissue and by comparing the ways of manifestation of a disease, cause of all mental diseases could be found. He proposed the first systematic classification of mental diseases in the Handbook of Psychiatry. Thanks to Dr. Kraepelin, who distinguished this “new” disease from the historically known senile dementia in 1910 (in the 8th edition of his Handbook of Psychiatry, the chapter on Senile and Presenile Dementias), the disease has been called Alzheimer (’s) disease (AD) (Verhey, 2009).

## 2. Alzheimer’s disease: generalities

The precise onset of clinical AD is very difficult to discern by both patient and family. The earliest symptoms are often manifested as subtle, intermittent deficits in the

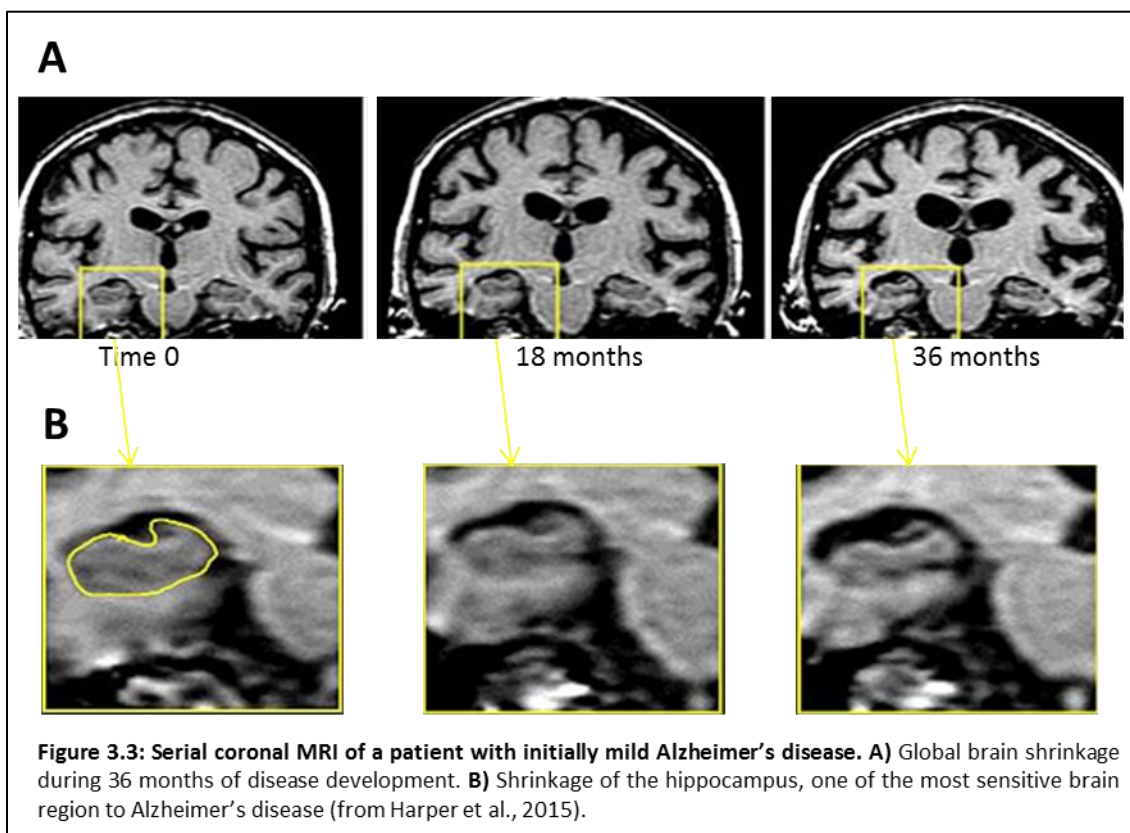
remembrance of events of everyday life, referred to as loss of episodic memory. Early warning signs are frequently dismissed as normal aspects of aging, but as memory loss slowly accelerates, the potential gravity of the situation becomes apparent. After many months of progressive impairment, first of declarative and then also of non-declarative memory, other cognitive symptoms appear and slowly progress. Over a further period of years or even a decade or more, a profound dementia develops and affects multiple cognitive and behavioral spheres and is often accompanied by extrapyramidal motor signs, slowed gait, and incontinence (Romanelli et al., 1990; Morris and Rubin, 1991). Death usually comes by way of minor respiratory complications, such as aspiration or pneumonia, often in the middle of the night.

Pathologically, the AD brain at end stage is characterized by **atrophy of the hippocampal formation and cerebral cortex and ventricular enlargement**, all greater than expected for the patient's age. Microscopically, there is a **decrease in the number of neuronal cell bodies** in the limbic and association cortices and in certain subcortical nuclei that project to them (Khachaturian, 1985; Gomez-Isla et al., 1997; Uylings and de Brabander, 2002). **The most obvious microscopic changes in the AD brain are the senile amyloid plaques and neurofibrillary tangles (NFTs)** (Terry, 1963; Kidd, 1964). These two types of lesions usually occur in very substantial numbers in the hippocampus, amygdala, association cortices, and certain subcortical nuclei, and they are often accompanied by variable numbers of amyloid-bearing meningeal and cortical microvessels (i.e. congophilic amyloid angiopathy). Staining of AD brain sections with silver-protein solutions like Bielschowsky's silver impregnation reveals the NFTs and the abnormal axons and dendrites (dystrophic neurites) that surround many of the amyloid plaques and which are also scattered widely in the cortical neuropil. By electron microscopy, NFTs and some dystrophic neurites can be seen to contain bundles of paired helically wound 10 nm filaments (PHFs) intermixed with some straight 10 nm filaments. The highly insoluble filaments of the NFTs may be left behind as "ghost tangles" following the death of the neurons in which they originally formed (Braak and Braak, 1994). The principal subunit of the PHFs is the microtubule-associated protein tau, which undergoes hyperphosphorylation and detachment from microtubules to form these abnormal filaments (Lee, 2001; Mandelkow et al., 2003).

## B. Pathological hallmarks

### 1. Brain atrophy

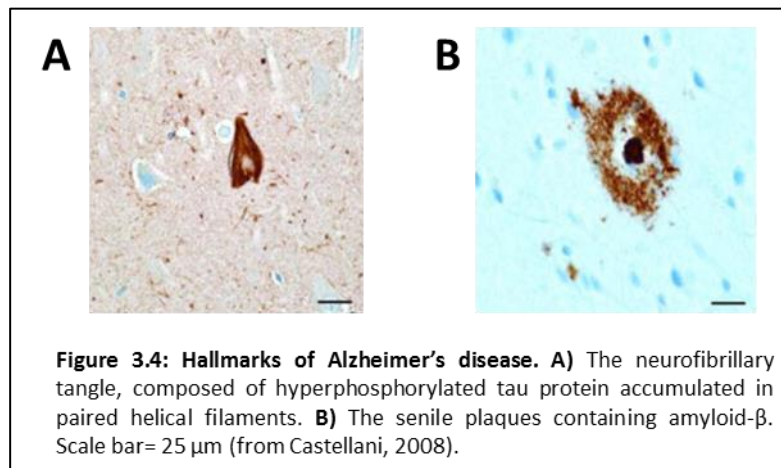
Loss of synapses and neurons, which extensively occurs in AD, results in gross atrophy (diminished cortical thickness in vulnerable regions and decreased of their volume) of the affected brain regions. In comparison with healthy adults and non-affected family members, studies using magnetic resonance imaging (MRI) have reported reductions in the size of specific brain regions of patients carrying APP or PS1 mutation (see below paragraph: Pathophysiology of Alzheimer's disease), as they progressed from presymptomatic through mild cognitive impairment to final diagnosis of AD (Figure 3.3). The study by Ridha et al. in 2006 found a significant reduction in mean hippocampal volume in mutation carriers as early as 3 years before clinical diagnosis, and a decreased mean brain volume 1 year before diagnosis. As mutation carriers moved through the clinical stages of the disease, both total hippocampal and whole-brain mean atrophy rates correspondingly increased (Ridha et al., 2006; Harper et al., 2015).



## 2. Amyloid plaques and neurofibrillary tangles

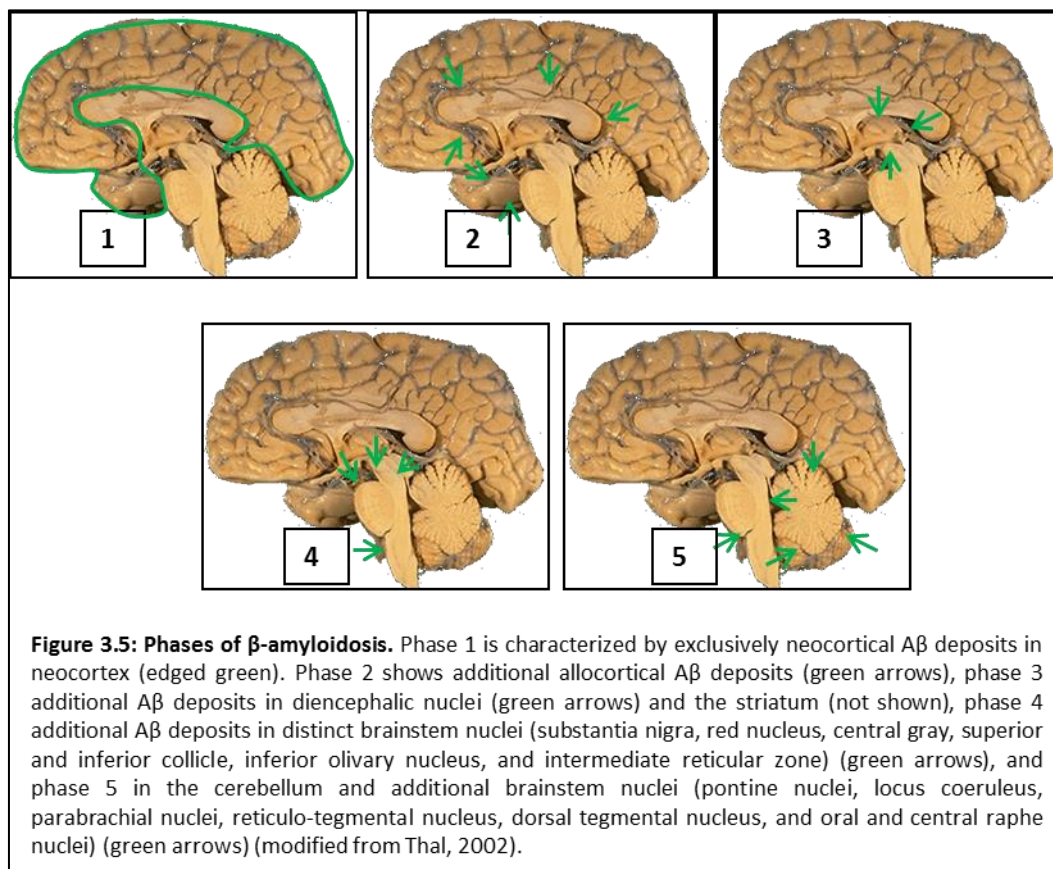
### a) Amyloid plaques

The AD brain is characterized microscopically by the combined presence of two classes of abnormal structures, extracellular amyloid plaques and intraneuronal NFTs (Bloom, 2014) (Figure 3.4).



Overproduction of amyloid  $\beta$  ( $A\beta$ ), especially  $A\beta_{1-42}$ , in AD patients, and possible extenuation of clearance, leads to spontaneous aggregation of the protein. A consequence of this process is the generation and accumulation of highly insoluble, densely packed filaments-plaques. In this formation, amyloid fibrils are resistant to proteolytic degradation (Ghiso and Frangione, 2002). In order to set a definite diagnosis of AD, it is necessary to detect plaques postmortem; otherwise it leads to exclusion of Alzheimer's as a diagnosis and consideration of frontal-temporal dementia or some other cause for the observed cognitive symptoms (McLean et al., 1999; Rodrigue et al., 2009; DaRocha-Souto et al., 2011). Accumulation of  $A\beta$  plaques can be divided into 5 phases, according to their temporal occurrence (Figure 3.5). Phase 1 is characterized by the appearance of plaques at the frontal, parietal, temporal and occipital cortices. These deposits are present in small groups, diffusely, in the cortical layers II, III, IV and V. In Phase 2, the deposits affect the entorhinal region, CA1 region of the hippocampus, insular cortex, amygdala and cingulate gyrus. Phase 3 is characterized by an invasion of deposits in subcortical regions namely the caudate, putamen, thalamus, hypothalamus including mammillary bodies, lateral habenular nucleus and white matter. During phases 4 and 5, much of the brain structures, from the substantia

nigra to the cerebellum are affected by amyloid deposits. The phases of  $\beta$  amyloidosis correlate significantly with the evolution of neurofibrillary lesions (Thal et al., 2002).



### ***b) Neurofibrillary tangles***

Under normal conditions, tau protein has a role in the polymerization of tubulin into microtubules and their stabilization. The role of tau in microtubule stabilization is important for the establishment and maintenance of axonal transport (Drubin et al., 1986). In contrast, the cytosolic abnormally hyperphosphorylated tau does not interact with tubulin/microtubules. The hyperphosphorylation of tau results from both an imbalance between the activities of tau kinases and tau phosphatases as well as changes in tau's conformation which affect its interaction with these enzymes (Iqbal et al., 2005). In pathological conditions, abnormally phosphorylated at certain residues, tau loses its solubility property and forms intracellular filamentous structures-neurofibrillary tangles (NFTs). NFTs are the major microscopic lesions of AD and are located mainly in pyramidal neurons of the hippocampus and in the neocortex (Braak and Braak, 1991).

As observed in amyloid plaques, the growth of NFTs also has a specific pattern. The first affected regions in the brains from patients with dementia, compared to healthy persons, are the trans-entorhinal cortex and hippocampus and later, NFTs occupy the whole neocortex (Braak and Braak, 1991). The same study has identified that the growth of NFTs in the brain gradually develops in six stages: entorhinal stage I and II (NFTs in the trans-entorhinal cortex and in the entorhinal cortex), limbic stages III and IV (extension to the hippocampus, then in the subiculum), and iso-cortical stages V and VI.

### **3. Neuroinflammation**

Besides the typical neuropathological hallmarks of AD, amyloid plaques and NFTs, neuroinflammation also is evident in the brains of AD patients. It is characterized by clusters of activated microglia and astrocytes around amyloid plaques, with the presence of inflammatory cytokines: IL-1, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Griffin et al., 1998; Takeda et al., 2014).

In the healthy brain, microglia is the main form of active immune defense. Although glial cells display neurotrophic properties, their potential neurotoxic roles have been investigated in AD research. As shown in in vitro studies, microglia after activation may produce a variety of pro-inflammatory mediators and potentially neurotoxic substances such as proteins from the complement system, cytokines, reactive oxygen intermediates, secreted proteases, excitatory amino acids, and nitric oxide. These mediators could further contribute to localize or even more widespread CNS injury (Akiyama et al., 2000).

### **4. Evaluation of cognitive decline**

Deficits in cognitive functions gradually develop over time in patients with AD. Roughly, cognitive decline could be divided in 7 stages, from the first stage, which corresponds to normal cognitive function, to the final 7<sup>th</sup> stage, when individuals lose the ability to react with their surroundings, to carry on a conversation and to control movement. Summary of the stages is given in Figure 3.6 (from [http://www.alz.org/alzheimers\\_disease\\_stages\\_of\\_alzheimers.asp](http://www.alz.org/alzheimers_disease_stages_of_alzheimers.asp)).



Stage	Name	AD stage	Symptoms	Detection/ independence of patient
<b>I</b>	No impairment	Normal function	Subjectively and objectively normal	Independent
<b>II</b>	Very mild cognitive decline	Normal age-related changes or earliest signs of AD	Forgetting familiar words or the location	Cannot be detected during examination/ Independent
<b>III</b>	Mild cognitive decline	Early-stage AD	Problems with right word/name, difficulties in performing tasks, losing/misplacing valuable objects, trouble with planning/organizing	May be able to detect problems in memory or concentration
<b>IV</b>	Moderate cognitive decline	Mild or early-stage AD	Forgetfulness of recent events and someone's personal history, becoming moody/withdrawn, greater difficulties in performing tasks	Clear-cut deficits on interview/ Might live independently or with assistance
<b>V</b>	Moderately severe cognitive decline	Moderate or mid-stage AD	Unable to recall own address/phone number, grandchildren's name, confused about place and time, cannot choose appropriate clothing	Need help with day-to-day activities
<b>VI</b>	Severe cognitive decline	Moderately severe or mid-stage AD	Lose awareness of recent experience, personal history, need help for dressing, changes in sleeping patterns, trouble controlling bladder or bowels, behavioral changes (delusions, repetitive behaviors, agitation)	Mostly necessary institutional care
<b>VII</b>	Very severe cognitive decline	Severe or late-stage AD	Lose ability to respond to the environment, inability for movement control, reflexes abnormal, swallowing impaired	Complex care

**Figure 3.6: Summary of the stages of Alzheimer's disease and its major symptoms** (modified from [http://www.alz.org/alzheimers\\_disease\\_stages\\_of\\_alzheimers.asp](http://www.alz.org/alzheimers_disease_stages_of_alzheimers.asp)).

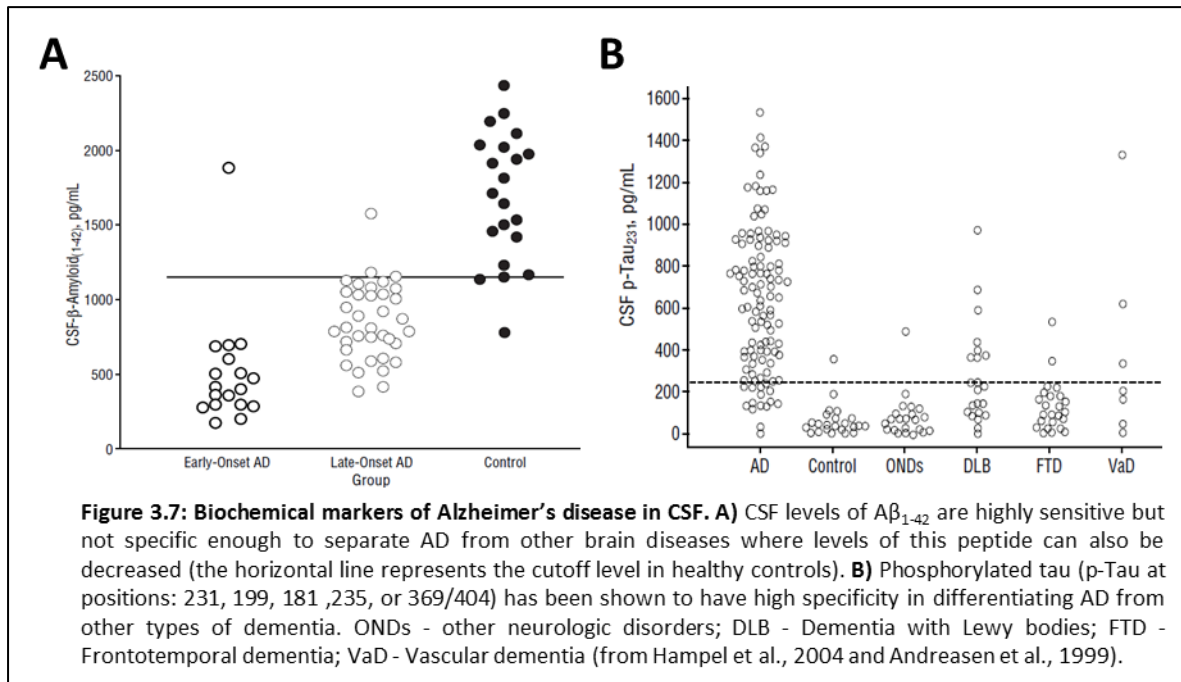
Doctors have developed a quick and short test, the Mini Mental State Examination (MMSE), which can serve as a screening tool for evaluating cognitive functions in patients possibly affected with AD. It measures the patient's time and space orientation, immediate recall, short - term verbal memory, calculation, language, and construct ability. It includes 11 questions, requires 5-10 minutes to be performed and is practical for serial and routine

use. The best (maximum) score is 30 points (Folstein et al., 1975). However, this test is not meant to provide a diagnosis of a disease. In order to gain a precise diagnosis, it is necessary to expand search into the patient's history and to perform physical examination and laboratory tests, including EEG, MRI and PET scan.

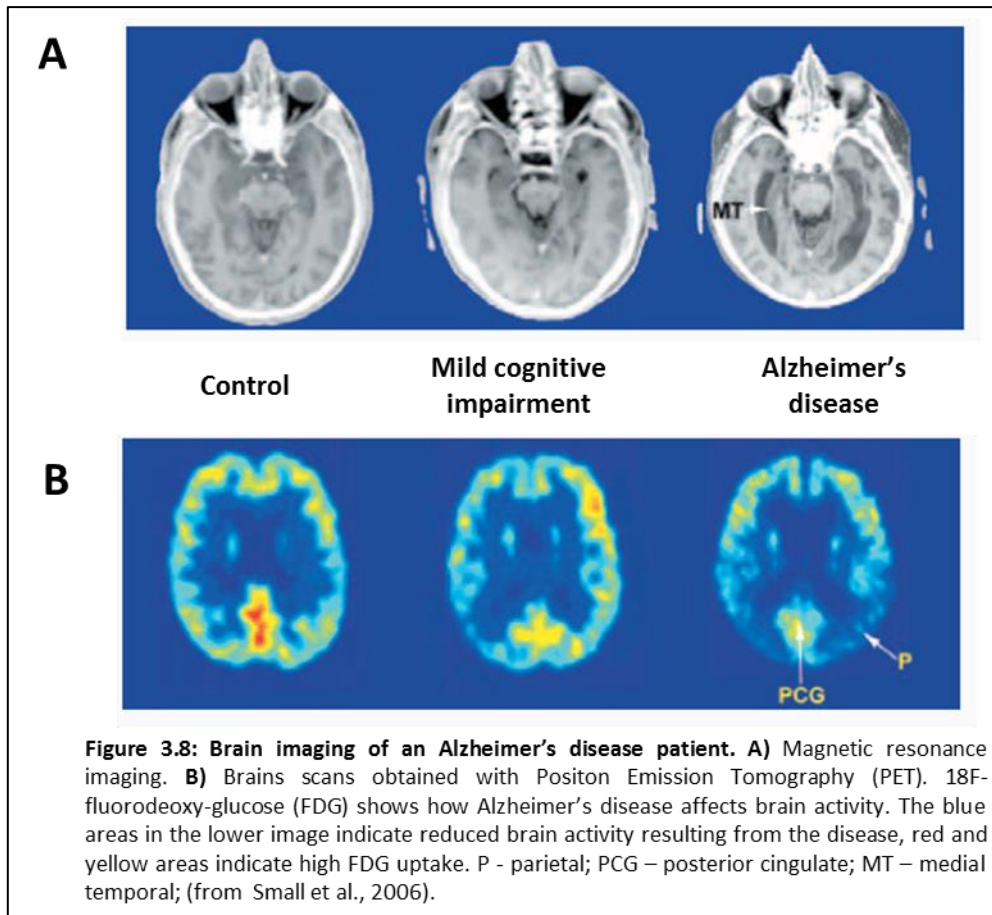
## 5. Biomarkers of the disease

Biomarkers are biological indicators used for identification of physiological changes, for monitoring of disease progression, and for evaluating a therapy response. Properties of pertinent biomarkers include high sensitivity and specificity, non-invasiveness, possibility for repeated measurements, availability, simplicity to perform and acceptable cost.

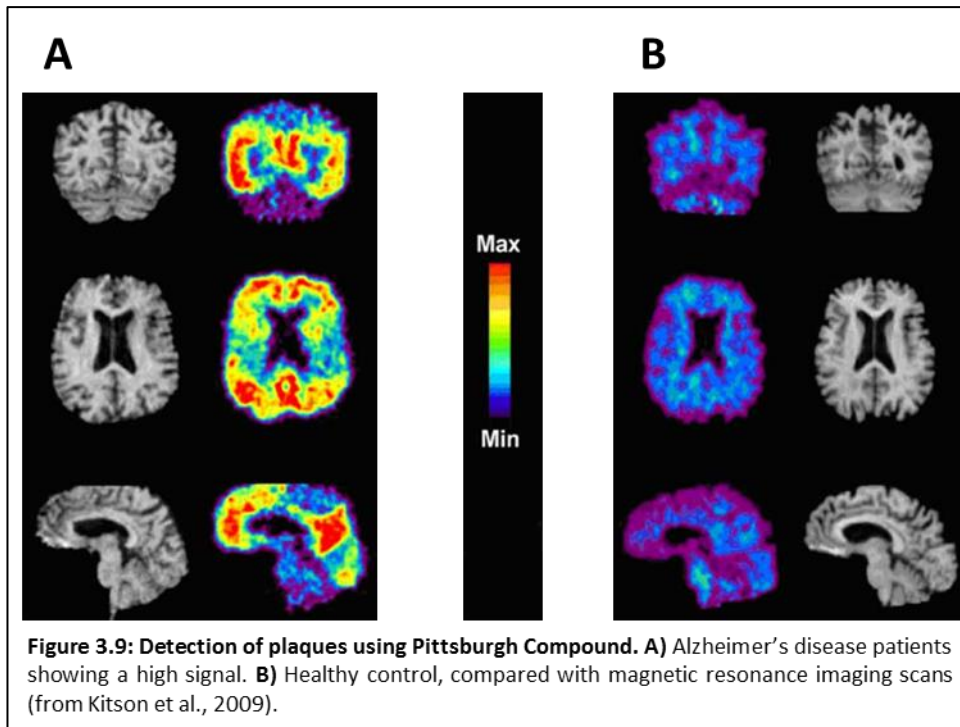
AD biomarkers, currently in use, are measured in the cerebrospinal fluid (CSF) or obtained via brain imaging methods. Since most of the processes that occur in the brain reflect in the CSF, CSF punctation and then biochemical analyses represents a good method for early detection of the brain changes, although it has disadvantages such as invasiveness. Currently tested proteins from CSF for detection and monitoring of AD are  $A\beta_{1-42}$ ,  $A\beta_{1-40}$ , total tau (t-Tau) and phosphorylated tau (p-Tau) proteins.  $A\beta_{1-42}$ , the toxic form of amyloid precursor protein (APP) degradation, analyzed by enzyme-linked immunosorbent assay (ELISA) showed significant decrease in CSF compared to healthy individuals. This decrease could be explained by its deposition within plaques (Grimmer et al., 2009). CSF levels of  $A\beta_{1-42}$  are highly sensitive but not specific enough to separate AD from other brain diseases where levels of this peptide can also be decreased. To generate more specific results, it is better to use  $A\beta_{1-42}/A\beta_{1-40}$  ratio (Blennow and Hampel, 2003). In contrast to  $A\beta$  levels, expression of tau protein increases in CSF during the course of AD. Many studies confirmed a great increase of phosphorylated form of tau in AD patients compared to healthy individuals, possibly as a reflection of intensive neuronal degradation. p-Tau (at positions 231, 199, 181, 235, 369/404) has been shown to have a high specificity (more than 75%) in differentiating AD from other types of dementia (Hampel et al., 2004) (Figure 3.7). Combination of tau with  $A\beta_{1-42}/p\text{-Tau}181$  resulted in even higher specificity (Hansson, 2006). In addition to these, there are new biomarkers for monitoring AD, such as BACE1 activity, sAPP $\alpha/\beta$ , neprilysin, cystatin C,  $\alpha$ -dystroglycan, GDNF, BDNF, IL-1, IL-6, and TNF- $\alpha$  (Babic et al., 2014).



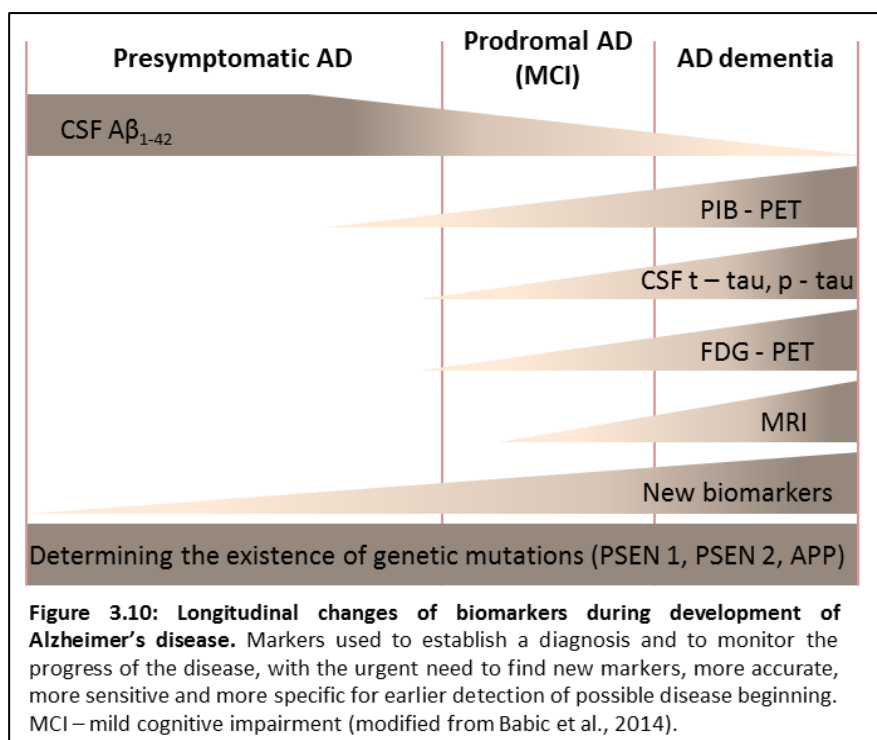
Brain imaging techniques are non-invasive methods with high specificity and sensitivity, but expensive and not readily available. Magnetic resonance imaging (MRI) is a structural imaging technique that can visualize reduction in overall brain volume, increased ventricular volume and atrophy of the medial temporal lobe regions in patients suffering from AD (Apostolova et al., 2006) (Figure 3.8).



The sole application of this technique is not sufficient for the diagnosis of AD (van de Pol et al., 2006). Functional MRI (fMRI) measures brain activity by detecting changes in blood flow, but it is still not applicable for diagnosis of AD due to high variability. The reduction in metabolism and cerebral perfusion in patients with AD may be assessed by the positron emission tomography (PET) imaging method (reduced cortical 18F-fluorodeoxyglucose uptake) (Kawachi et al., 2006), and by the single photon emission computed tomography (SPECT) scan (Trollor et al., 2005). These changes are observed in specific areas such as the temporo-parietal junction, the temporal, parietal and frontal lobes as well as in the hippocampal formation and the posterior cingulate cortex. Further development of PET radiotracers led to detection of plaques (with PiB - Pittsburgh Compound B) (Mason and Mathis, 2003) (Figure 3.9), or plaques and tangles (with FDDNP (2-(1-{6-[(2-[18F] fluoroethyl) (methyl) amino]-2-naphthyl} ethylidene) malononitrile) (Small et al., 2006). Combination of plaques imaging with MRI showed very high specificity and sensitivity.



Markers used to establish a diagnosis and to monitor the progress of AD are summarized in Figure 3.10. There is still an urgent need to find new reliable markers, more accurate, more sensitive and more specific for earlier detection of the disease and in order to establish the correct diagnosis and treatment, bearing in mind that AD can develop 10-20 years before the first symptoms.



## C. Classification of Alzheimer's disease

AD can be classified according to the onset or the origin of the disease. The most common form of AD is the late onset form that is defined as AD with an age onset later than 65 years, while early-onset AD accounts for approximately 1% to 6% of all cases and age onset ranges roughly from 30 years to 65 years. Genetically, AD is usually divided into familial cases with Mendelian inheritance (Familial AD or FAD) and sporadic cases with no familial aggregation. Familial cases are predominantly early-onset even if late onset cases have been described (Bekris et al., 2010).

### 1. Sporadic forms of AD

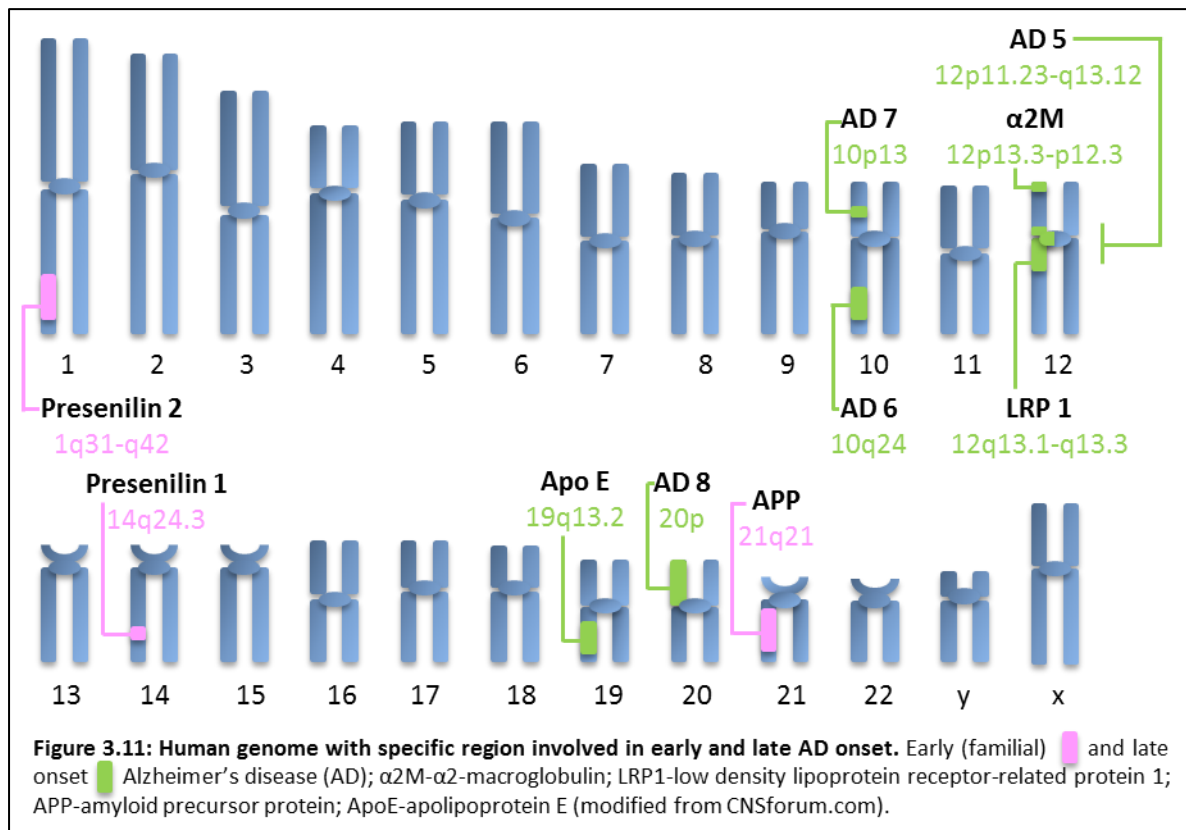
About 95% of all cases of AD are considered as sporadic forms of the disease. Characteristic is a late-onset of the disease (>65 year-old), with an incidence significantly increasing with age. **The reasons for the occurrence and progression of this sporadic form are still unknown**, but a number of factors may increase or decrease the person's chances of developing the disease. These risk factors include age, genetics, environment, and lifestyle. The importance of these factors may vary according to the person. Some risk factors can be changed or controlled while others cannot.

After the age, the best-documented risk factor for the development of AD is the **apolipoprotein E (ApoE) genotype**. This gene encodes a 299-aminoacid lipoprotein which binds amyloid proteins. Specifically, ApoE exists in three common polymorphic forms (epsilon 2 ( $\epsilon 2$ ), epsilon 3 ( $\epsilon 3$ ) and epsilon 4 ( $\epsilon 4$ )) and one of them,  $\epsilon 4$ , is strongly associated with an increased risk of AD, not only in a homozygous state, but also in a heterozygous one. Persons with one  $\epsilon 4$  allele have been reported to have ~3 times more chances to develop Alzheimer's disease than those with an ApoE  $\epsilon 3/\epsilon 3$  genotype. The risk of developing AD for the persons with both  $\epsilon 4$  alleles increases up to ~13 times compared to individuals with an ApoE  $\epsilon 3/\epsilon 3$  genotype (Lindsay et al., 2002; Klages et al., 2003). It is also reported that the ApoE  $\epsilon 4$  allele accelerates the onset of the familial form of AD in patients with APP but not with presenilin 1 mutations (van Duijn et al., 1994). However, over 60% of all sporadic cases are not associated with ApoE, suggesting that other genetic and environmental factors may contribute to the disease. These factors can be divided into **external and internal**. External factors include the behavior of an individual and the environmental impact, for example:

alcohol abuse, smoking, malnutrition, traumatic head injury, poor education or mental and leisure inactivity. The internal factors can include a variety of diseases already developed such as depression, Down syndrome, hormonal disorders, cardiovascular diseases and related vascular risk factors (hyperlipidemia, hypertension, diabetes).

## 2. **Familial forms of AD**

Familial forms of AD are diagnosed in families that have more than one member with AD (usually multiple affected persons in more than one generation), but it is clinically indistinguishable from the sporadic form, except that people manifest the disease in their late 30s, 40s, or 50s. This early-onset dominantly inherited AD represents only 5% of all AD cases and is due to mutations in the APP, presenilin1 and/or presenilin 2 genes (see below paragraph: Pathophysiology of Alzheimer's disease). Those mutations are inherited through an autosomal dominant way with complete penetrance. At the 21<sup>st</sup> chromosome, at least 33 different pathogenic mutations of APP have been identified so far, as nucleotide variations within the domain which encodes amyloid  $\beta$  sequence or out of it. At the 14<sup>th</sup> chromosome, which carries the gene encoding presenilin 1, there is more than 180 pathogenic mutations, while at the chromosome number 1, with the gene encoding presenilin 2, less than 15 mutations have been reported ([www.molgen.ua.ac.be/ADMutations/](http://www.molgen.ua.ac.be/ADMutations/)). The determination of the mutations responsible for the disease in these several hundred families is an important step to better understand the molecular pathogenesis of AD (Figure 3.11).



## D. Socio-economic challenge

### 1. Epidemiology

Dementia and particularly AD is closely linked to aging. In AD, the prevalence rate doubles every 5 years and various epidemiological studies have shown the exponential growth of the prevalence rate with age, starting from around 1.5-2.5% in the 65-69 year, reaching almost 40% in the 90-94 year age group (Fratiglioni et al., 2001). In addition, according to the world health organization the proportion of people over 65 years is set to increase from 6.8% in 2000 to 16.2% by 2050. As a main consequence, the prevalence of dementia is expected to rise to 81 million by 2040, assuming no changes in incidence, mortality and current preventive or curative treatments are found for neurodegenerative disorders (Ferri et al., 2005).

### 2. Alzheimer's disease: a social and economic challenge

**AD has a strong unfeigned medical, social, psychological, and financial impact on patients, their families, caregivers, and their surroundings.** Persons with AD require "formal" medical services (professional caregivers, nurses, hospitalization, and



institutionalization) and “informal” services, mostly provided by family members (Rice et al., 1993). Patients are usually placed in special institutions when the disease has progressed significantly and when 24-hour supervision is required. Expenses necessary for caring of patients can be divided as direct and indirect costs. Direct costs involve hospitalization, physician visits, paid home care, medicaments, laboratory tests, and institutionalization. The largest consumer of financial means is institutionalization. Indirect costs may then include emotional and psychological consequences for families such as stress, sleeplessness, fatigue, anxiety, social isolation, and poor quality of life, as well as associated medical consumption (Rigaud et al., 2003; Llanque et al., 2014).

In 2010, the worldwide societal costs for AD and other dementias were estimated at \$604 billion, which constitutes approximately 1% of the aggregated global gross domestic product (Wimo et al., 2013) with the greatest proportion of costs from high-income countries. As an example, in USA, dementia is the most expensive disease, costing families and society \$157 billion to \$215 billion a year (Hurd et al., 2013). A simulation made in the UK in 2003 indicated that the cost of long-term care for patients with cognitive problems should rise from 6.9 billion euros in 1998 to 16.4 billion in 2031. This represents a 240% increase. Given the projected future numbers of individuals with dementias, the potential economic challenges ahead are clear. Today, about 36 million people worldwide suffer from AD and other dementias. This figure is projected to more than double by 2030 and reaches about 115 million in 2050 unless there are major improvements in prevention and/or cure. Overall, these predictions make AD and other dementia a major social and economic challenge for all industrialized countries in which lifespan has continuously increased over the last century (Alzheimer Disease International. World Alzheimer Report 2011. The benefits of early diagnosis and intervention. Published by Alzheimer’s Disease International (ADI), September 2011).

## **E. Pathophysiology of Alzheimer’s disease**

AD is a highly prevalent neurodegenerative disorder characterized by a progressive loss of cognition associated with the presence of two hallmark lesions, senile plaques and neurofibrillary tangles (NFTs), which result from the accumulation and deposition of the amyloid  $\beta$  peptide and the aggregation of hyperphosphorylated tau protein, respectively.

## 1. **From APP to $\beta$ -Amyloid Plaques**

Since its identification in senile plaques, A $\beta$  has been considered to play a central role in the pathology of AD. A $\beta$  is thought to accumulate in AD because of an imbalance in the production and clearance of this peptide resulting in the formation of the characteristic amyloid plaques in specific brain regions.

### ***a) From A $\beta$ to APP***

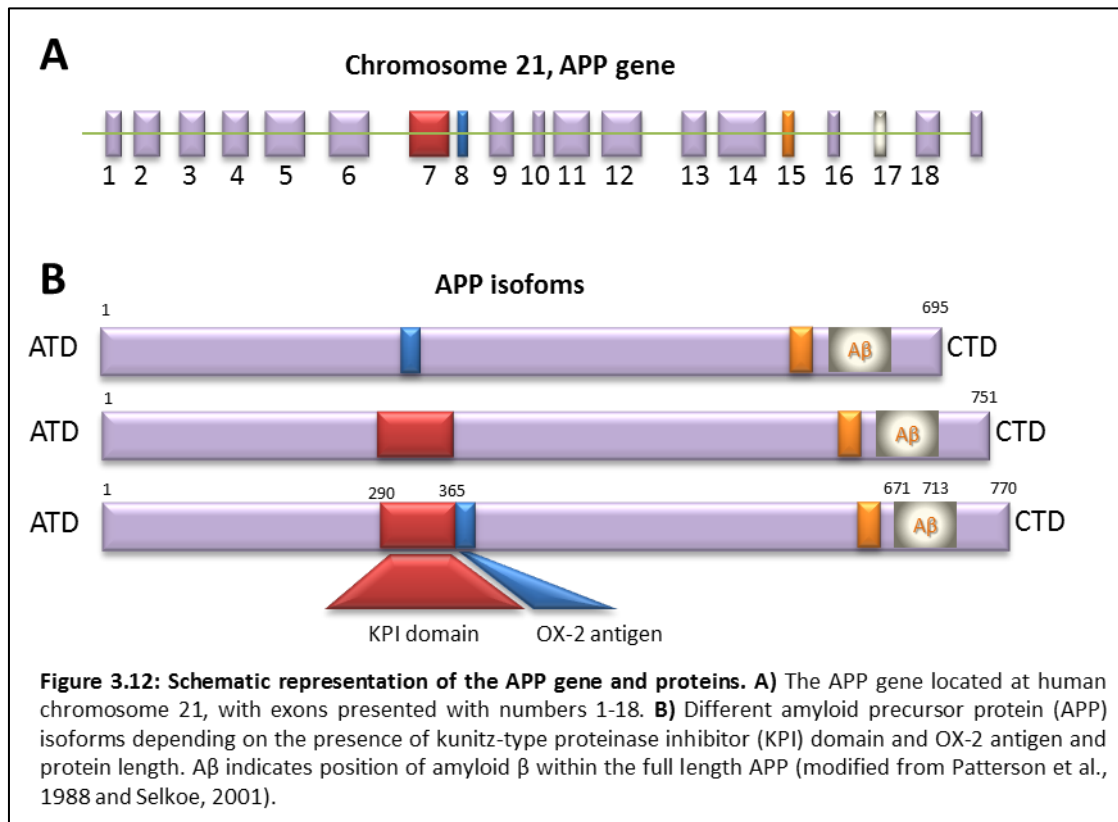
In the '80s, despite nearly eight decades that had lapsed since Dr. Alois Alzheimer introduced the disease, there was still nothing known about the amyloid etiology. In 1984, the group of Georges Glenner reported the amino acid sequences of the main component of A $\beta$  based on its analysis of cerebrovascular amyloid proteins derived from patients with Down syndrome. The isolation and sequencing of A $\beta$  protein by Georges Glenner and Caine Wong provided a turning point for research on the fundamental mechanisms of AD (Glenner and Wong, 1984). Indeed, based on A $\beta$  peptide sequence, the amyloid precursor protein gene was cloned by four laboratories and was found on chromosome 21 (Goldgaber et al., 1987a; Robakis et al., 1987; Tanzi et al., 1987).

### ***b) Amyloid precursor protein***

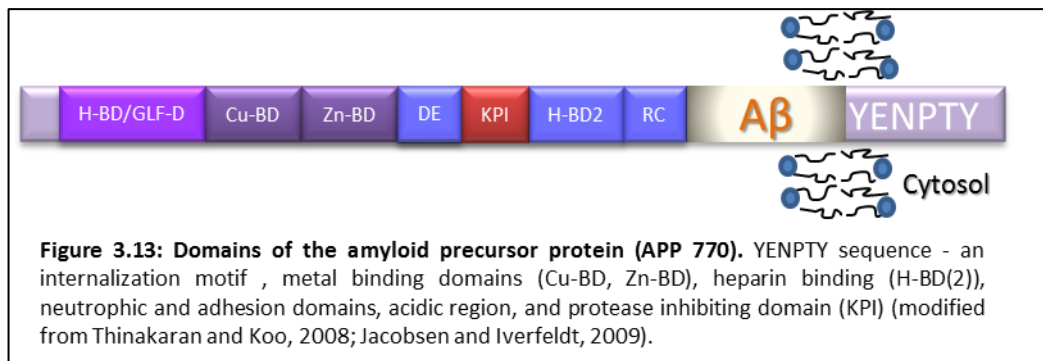
APP is encoded by a single gene of 18 exons spanning more than 170 Kb on chromosome 21 at loci 21q21 and 21q22 (Patterson et al., 1988) (Figure 3.12A). The region encoding the A $\beta$  sequence comprises part of exons 16 and 17 and is composed of 40 to 43 amino acid residues that extend from the ectodomain into the transmembrane domain of this protein. Human APP belongs to an evolutionarily conserved family of type I transmembrane glycoproteins that also includes the paralogs: amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2), which show functional redundancy but lack the A $\beta$  sequence (Goldgaber et al., 1987b).

Exons 7, 8, and 15 of the APP gene can be alternatively spliced to produce multiple isoforms. In the brain, the major isoform transcripts result from the splicing of exons 7 and 8, which gives rise to APP<sub>695</sub>, APP<sub>751</sub>, and APP<sub>770</sub> (Selkoe, 2001b). APP<sub>770</sub> and APP<sub>751</sub> both contain a serine protease inhibitory domain KPI encoded by exon 7. KPI-APPs are mainly

expressed in the brain by astrocytes and microglia, whereas the KPI-deficient APP, APP<sub>695</sub>, is abundant in neurons (LeBlanc et al., 1991) (Figure 3.12B).



APP is a type I transmembrane protein, which consists of short intracellular CTD and long extracellular ATD. The multiple domains located within the extracellular portion includes a signal peptide (SP), a heparin-binding/growth-factor-like domain 1 (HPBD1), a copper-binding domain (CuBD), a zinc-binding domain (ZnBD), a Kunitz-type protease inhibitor domain (KPI), a second heparin-binding domain 2 (HPBD2), a random coil region (RC) and the Aβ sequence (Thinakaran and Koo, 2008; Jacobsen and Iverfeldt, 2009). The remaining region consists of the cytoplasmic tail of APP, including AICD and an internalization motif YENPTY (residues 682-687 of APP695) (Figure 3.13).



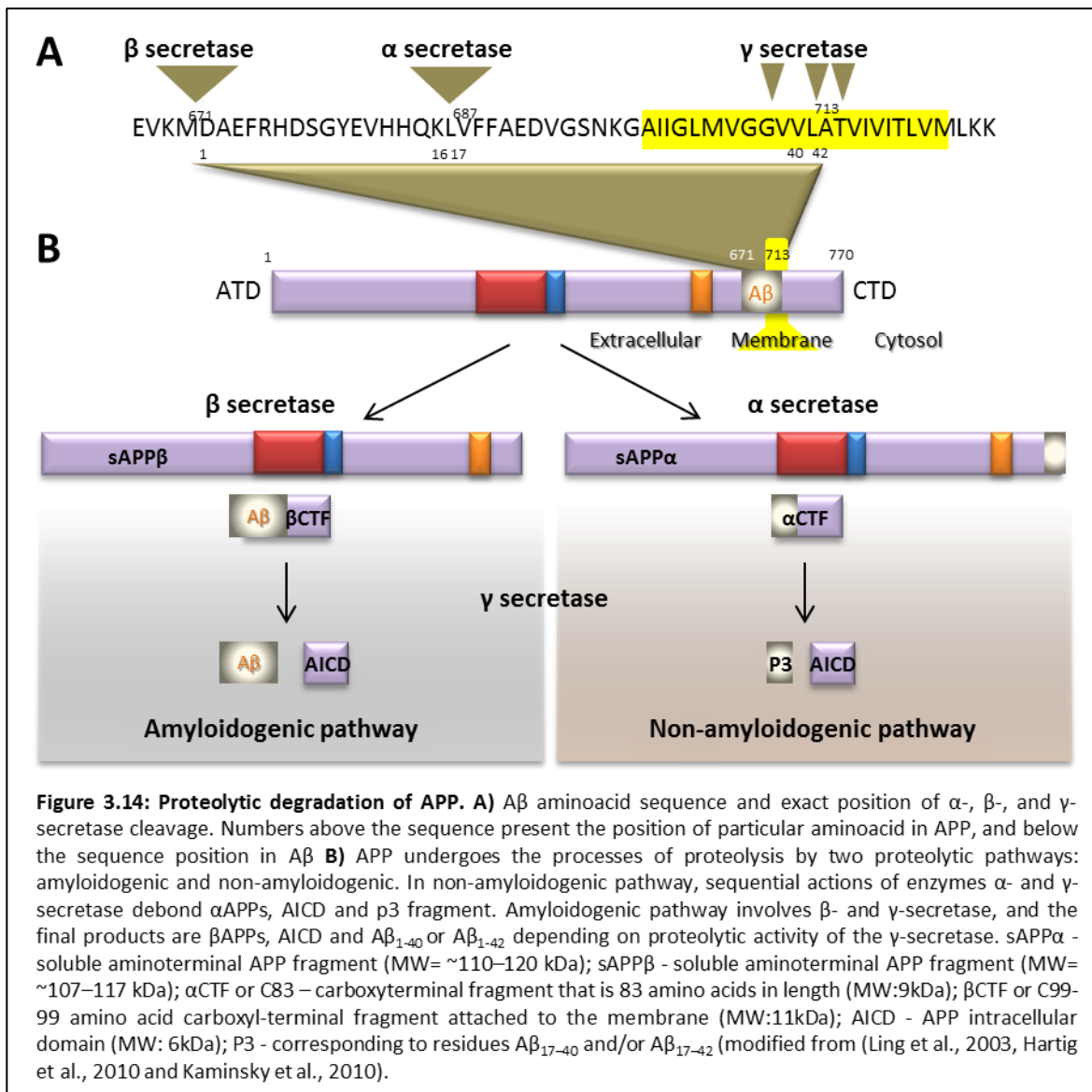
After the synthesis, it is post-translationally modified in Golgi complex and delivered to nerve endings by fast anterograde axonal transport (Koo et al., 1990).

Physiological role of APP is still not clarified, but some studies have shown that it is implicated in survival of neurons and neurite outgrowth, effects independent of the presence of the KPI domain (see below paragraph: Physiological role of APP products) (Araki et al., 1991; Hung et al., 1992).

### **c) APP processing**

APP can be processed in different ways by different sets of enzymes - one pathway leads to production of A $\beta$  (amyloidogenic), while another does not (non-amyloidogenic) (Figure 3.14). Usually, about 90% of APP enters the non-amyloidogenic pathway, and 10% the amyloidogenic one, but these ratios can change due to genetic or environmental factors, as well as with the aging of the individual. Cleavage products from both pathways may play important roles in neural development and function.

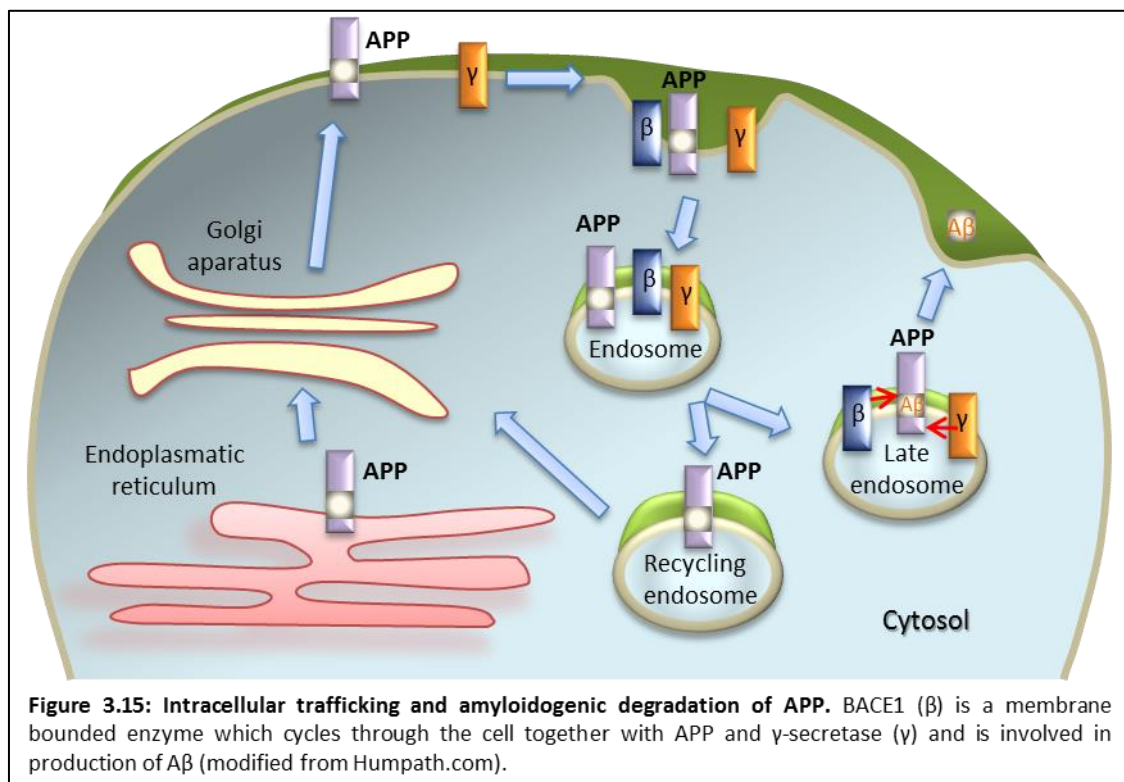
**In the non-amyloidogenic pathway,** APP is cleaved first by  $\alpha$ -secretase to yield a soluble amino-terminal fragment (sAPP $\alpha$ ) and a carboxyl-terminal fragment (CTF $\alpha$ ). sAPP $\alpha$  may be involved in the enhancement of synaptogenesis, neurite outgrowth and neuronal survival, and is considered to be neuroprotective (Ohsawa et al., 1997; Weyer et al., 2011). CTF $\alpha$  is retained in the membrane, where it is acted upon by presenilin-containing  $\gamma$ -secretase to yield a soluble amino-terminal fragment (p3) and a membrane-bound carboxyl-terminal fragment (AICD or APP intracellular domain). AICD may be involved in nuclear signalling via transcriptional regulation as well as axonal transport through its ability to associate with different proteins (Muller et al., 2007; Zhang et al., 2007b).



In the amyloidogenic pathway, APP is cleaved first by a different enzyme,  $\beta$ -secretase (a transmembrane aspartic protease), yielding a soluble amino-terminal fragment (sAPP $\beta$ ) and a membrane-bound carboxyl-terminal fragment ( $\beta$ CTF). This cut is made closer to the amino-terminal end of APP than with  $\alpha$ -secretase, making  $\beta$ CTF longer than  $\alpha$ CTF.  $\beta$ CTF is then acted upon by  $\gamma$ -secretase (as occurred in the previous pathway), yielding a membrane-bound carboxyl-terminal fragment (AICD) the same as before, and a soluble amino-terminal fragment (amyloid- $\beta$ , or A $\beta$ ) with different carboxyl terminus, including A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-42</sub> and other minor species (Ling et al., 2003; Hartig et al., 2010; Kaminsky et al., 2010) which is dependent of proteolytic activity of the  $\gamma$ -secretase. Additional cleavage at the amino-terminus of A $\beta$  results in A $\beta$ <sub>3-42</sub>, A $\beta$ <sub>11-40</sub> and A $\beta$ <sub>11-42</sub> (Lee et al., 2003). At least in the cerebrospinal fluid of non-demented controls, about one-half of the A $\beta$  ends at amino acid

40, 16% ends at amino acid 38, and 10% ends at amino acid 42 (Wiltfang et al., 2002; Bibl et al., 2012). However, A $\beta$ <sub>1-42</sub> is considered as the harmful peptide since it is much more prone to misfolding and builds up aggregates that are the key effectors of neurotoxicity (Walsh et al., 2002b; Wolfe and Guenette, 2007; Querfurth and LaFerla, 2010).

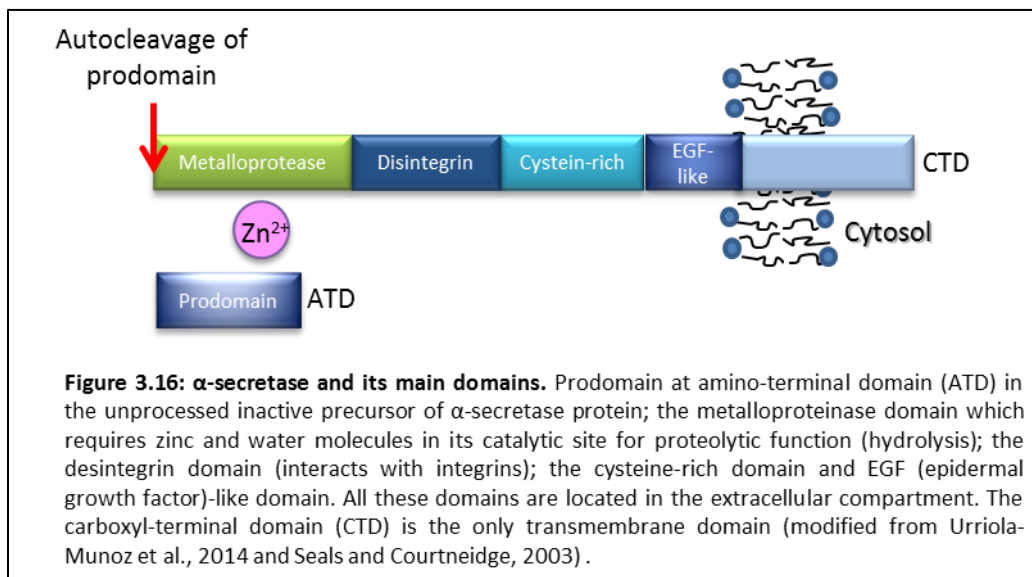
Although APP is expressed on the neuronal surface, it is mostly degraded in the intracellular compartment (Kuentzel et al., 1993; Caporaso et al., 1994). Moreover, blockade of endocytosis leads to an 80 % decrease of extracellular release of A $\beta$ . After its synthesis and posttranslational modification in Golgi complex, APP can be transported to the cell surface or to the endosomal compartment. On the cell surface, APP can be processed by  $\alpha$ - and  $\gamma$ -secretase, or internalized within several minutes into endosomal compartment, due to YENPTY domain, and degraded by  $\beta$ - and  $\gamma$ -secretase.  $\beta$ -secretase interacts with APP in endosomes and co-localized with the  $\gamma$ -secretase, while  $\alpha$ -secretase exerts its activity on the cell surface together with the  $\gamma$ -secretase (Kinoshita et al., 2003; Fukumori et al., 2006) (Figure 3.15).



## d) Secretases

### (1) $\alpha$ -secretase

**$\alpha$ -secretase** is a group of type I transmembrane proteins, “a desintegrin and a metalloproteinase”: ADAM 17 (TACE), ADAM 9 (MDC 9), ADAM 10 (Lammich et al., 1999; Allinson et al., 2003) and aspartyl protease: BACE 2. BACE2 is ubiquitously expressed in human tissue. It has longer CTD than other aspartyl proteases and requires autocleavage of prodomain for full enzymatic activity. It is proposed to function as an alternative  $\alpha$ -secretase, cutting APP mainly between A $\beta$  Phe20Ala21 and Phe19Phe20 (Yan et al., 2001), and as an antagonist for BACE1 ( $\beta$ -secretase) (Basi et al., 2003). ADAMs consist of a prodomain with ATD, a metalloproteinase domain, a desintegrin domain (to interact with integrins), a cysteine-rich domain and an EDF-like domain. The molecular weight of unprocessed precursor protein is 90-140 kDa. After maturation, which means removal of prodomain (~20kDa), it becomes an active enzyme (Urriola-Munoz et al., 2014). The metalloproteinase domain requires zinc and water molecules in its catalytic site as necessary components for proteolytic function (hydrolysis) (Seals and Courtneidge, 2003). The role of cysteine-rich domain and EDF-like domain has not been clarified yet but both domains might be regulator of metalloproteinase activity and interact with cell surface proteoglycans (Urriola-Munoz et al., 2014) (Figure 3.16).



ADAM10 is expressed in the brain (superficial cortical layers and in hippocampus), liver, thymus, spleen, and bone marrow (Marcinkiewicz and Seidah, 2000). Cleavage of APP

by the  $\alpha$ -secretase in extracellular compartment releases  $\alpha$ APPs (105-125kDa) and creates the membrane anchored  $\alpha$  carboxyl-terminal fragment ( $\alpha$ CTF) also called C83 (Weidemann et al., 1989). This proteolysis occurs within the A $\beta$  domain of APP after Lys16, so this cannot lead to production of toxic A $\beta$ .

## (2) $\beta$ -secretase

**$\beta$ -secretase** (70kDa) - also called BACE1 ( $\beta$ -site APP cleaving enzyme), memapsin or Asp2, is the type 1 transmembrane aspartic protease (Vassar et al., 1999). Amino-terminus of the enzyme faces the extracellular compartment and contains a prodomain, while the palmitoylated CTD-tail is located intracellularly (Hussain et al., 1999). After synthesis in the ER as a proBACE1 (60kDa), this enzyme undergoes a rapid maturation in Golgi complex which implies cleavage of the propeptide domain and glycosylation that may be important for enzymatic activity and palmitoylation may affect the trafficking and localization of BACE1 (Benjannet et al., 2001).

The gene for BACE1, localized on 11<sup>th</sup> human chromosome, occupies around 30 kb including 9 exons and 8 introns (Sambamurti et al., 2004). The promoter of BACE1 has a binding site for the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). Its activation with nonsteroidal anti-inflammatory drugs (NSAID) or PPAR $\gamma$  agonist leads to the repression of the BACE1 promoter activity, which further leads to reduced production of A $\beta$  (Sastre et al., 2003). Hence, long-term application of NSAID in animal models has led to the positive effects on A $\beta$  load (Lim et al., 2000; Lim et al., 2001).

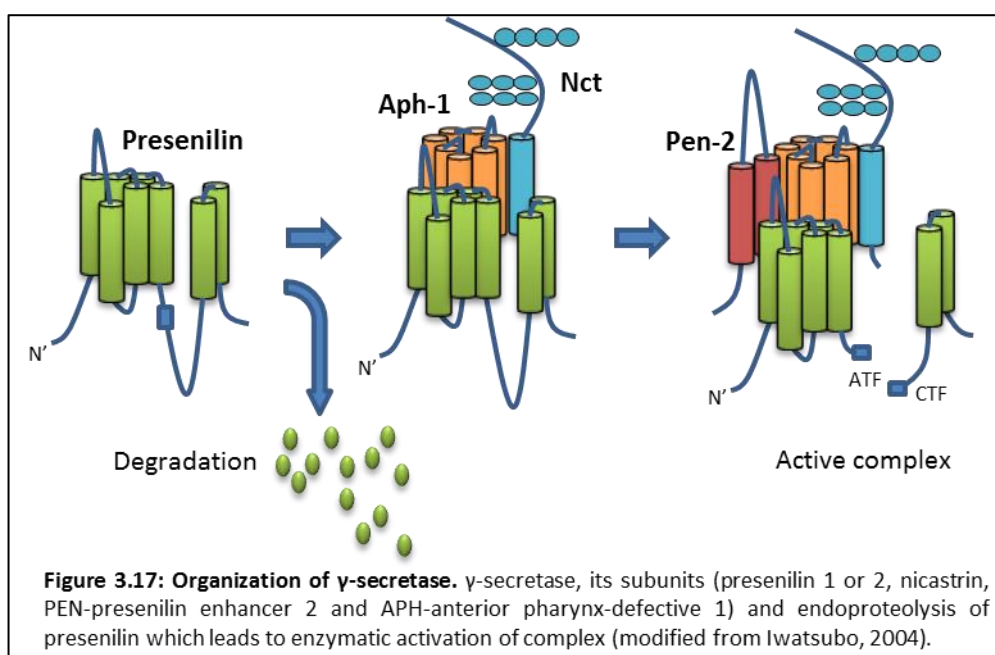
The soluble active BACE1 is also detected in the CSF, what gives the possibility for usage as an easy and fast diagnostic and prognostic tool. BACE1 is a membrane bounded enzyme with the acidic pH optimum ( $\sim$ 4.5) (Haass et al., 1993) which suggests that it is active in Golgi apparatus, trans-Golgi network and endosomes. It cycles through the cell, together with APP, in his active form (Marcinkiewicz and Seidah, 2000). This trafficking is controlled by dileucine motif of BACE1 CTD, whereas depletion or mutation of this motif cause the increased membrane surface level of this enzyme compared to the endosomes (Pastorino et al., 2002).



In the healthy brain, most of APP is cleaved by  $\alpha$ -secretase, and only a small part by  $\beta$ -secretase. Also when it is transfected in APP-overexpressing cells, BACE1 induces significant increases of  $\beta$ APPs, and decreases of  $\alpha$ APPs. This indicates a competition between these two enzymes for APP as a substrate (Vassar et al., 1999). Cleavage of APP by BACE1 (Vassar et al., 1999) releases  $\beta$ APPs in extracellular compartment and membrane bound fragment C99, which is further subject to cleaving by  $\gamma$ -secretase.

### (3) $\gamma$ -secretase

The final step in the processing of APP is further enzymatic digestion of  $\alpha$ - and  $\beta$ - CTF (C83 and C99) by  $\gamma$ -secretase to p3 and A $\beta$  peptide, respectively (Wolfe et al., 1999). This enzyme is a multiprotein complex which is expressed ubiquitously in brain and peripheral tissues and requires all its subunits to manifest the proteolytic activity. Those subunits are presenilin (PS) 1 or 2, nicastrin, presenilin enhancer 2 (PEN 2) and anterior pharynx-defective phenotype 1 (APH 1) (Takasugi et al., 2003; Iwatsubo, 2004). PS1 and 2 are catalytic subunits of the enzyme, whose mutation on related genes on chromosome 14 and 1 is linked with the early onset of AD (Thinakaran et al., 1996). Nicastrin (type 1 transmembrane protein) and APH 1 (7-time pass membrane protein) exert collaborative stabilization of PS holoprotein, while PEN 2 (2-time pass protein) leads to the final maturation by inducing endoproteolysis of PS at the domain in which around 58% of missense mutation, related with FAD, occurs (Takasugi et al., 2003).



Endoproteolysis of presenilin protein within the region encoded by exon 9, in cytosolic loop between transmembrane loops 6 and 7 (Wolfe et al., 1999), produces two stable fragments: the amino-terminal fragment (ATF: 27-28 kDa) and the carboxyl-terminal fragment (CTF: 16-17 kDa), which bind to each other (by non-covalent bond) to exert the physiological activity (ATF-CTF=1-1) (Figure 3.17).

Deletion of exon 9, which may be a consequence of missense or a point mutation (S290C), upstream of a splice acceptor site of presenilin 1 (PS1dE9), generates a mutant protein that exhibits similar physiological and biophysical properties, although it does not undergo the endoproteolysis (Thinakaran et al., 1996). The lack of this domain encoded by exon 9 allows the intact protein to act like the natural ATF-CTF complex, but with altered activity (Capell et al., 1998). Cleavage of membrane-tethered  $\alpha$ CTF (C 83) and  $\beta$ CTF (C 99) by  $\gamma$ -secretase complex within the transmembrane region further releases the AICD fragment in the intracellular, and p3 (3 kDa) and  $A\beta$  (4 kDa) in the extracellular compartment, respectively. Familial AD-linked mutations in PS1 and 2 cause favored cleavage at position 42 against 40 of  $A\beta$ , thus increasing  $A\beta_{1-42}/A\beta_{1-40}$  ratio (Thinakaran and Koo, 2008).

#### ***e) Physiological role of APP products***

While  $A\beta$  is central to AD pathogenesis, the evolutionary conservation of APP and the presence of APP isoforms lacking the  $A\beta$  sequence indicates that amyloidogenesis is unlikely to be the main physiological function of this protein family. Recent accumulative evidence demonstrated that APP is important for neuron generation, neuron differentiation as well as neural migration. Moreover, these APP products could exert a protective and/or neurotrophic effects in several pathological conditions.

#### ***f) Role of APP products in neurogenesis***

Neurons in the embryonic mammalian brain are generated in progenitor zones that line the ventricles. Soon after their birth, they undergo active cell migration to reach distant locations, where they eventually form neuronal circuits. As the developmental expression of APP corresponds to the timing of neuronal differentiation, it was postulated that APP or APP metabolites might play a role in the neuronal differentiation of neural progenitor cells. Indeed, in human embryonic stem cells, sAPP $\alpha$  treatment induced neuronal phenotype

(Freude et al., 2011) confirming this postulate. In contrast, treatment of human neural progenitor cells with APP causes a shift toward glial differentiation (Sugaya, 2008). If these results highlight the importance of APP metabolites in cell lineage commitment, the role of specific APP metabolites in the differentiation of neural progenitor cells is still a matter of debate.

While the neural progenitor cells generate new neurons in the mammalian brain during the embryonic life, they also generate new neurons in the adult brain. The formation of new neurons or astroglia in the adult brain is a multifaced process including the necessity of a niche that supports continuous proliferation of neural progenitor cells, the determination of neural lineage, migration of immature cells and functional incorporation into existing neural networks. Since initial evidence for adult neurogenic involvement in memory formation in songbird (Nottebohm, 2004), several studies in mammals using different strains of mice (Kempermann et al., 1997a), environmental enrichment (Kempermann et al., 1997b) and running (van Praag et al., 1999) have shown a correlation between increases in adult neurogenesis and enhanced performance on a spatial memory tasks. Because of this last point, the role of APP products in neurogenesis has been largely documented and many of the metabolites of APP have been involved in one or more of the neurogenic steps.

**Remark:** The miscleavage of APP would readily impact developmental and postnatal neurogenesis which contributes to cognitive deficits characterizing AD.

#### (1) Neuroprotective role of sAPP $\alpha$

There is substantial experimental evidence for a neuromodulatory and/or a neuroprotective function of sAPP $\alpha$  in neuronal cells and primary neurons *in vitro* (Araki et al., 1991; Mattson et al., 1993; Schubert and Behl, 1993; Bowes et al., 1994; Goodman and Mattson, 1994; Roch et al., 1994; Furukawa and Mattson, 1998; Guo et al., 1998; Xu et al., 1999; Luo et al., 2001; Kogel et al., 2003; Esposito et al., 2004; Stein et al., 2004; Han et al., 2005; Gralle et al., 2009), but the exact molecular mechanisms underlying these neuroprotective effects remain to be clearly defined. *In vivo* evidence for a protective role of APP is sparse. However, APP upregulation has been reported following brain injury in mammals and *Drosophila* (Murakami et al., 1998; Van den Heuvel et al., 1999; Ramirez et al.,

2001; Leyssen et al., 2005) and injection of sAPP $\alpha$  into the brain of rats with traumatic brain injury results in beneficial effects on motor or cognitive outcome (Thornton et al., 2006; Corrigan et al., 2011). In a similar fashion, sAPP $\alpha$  was also shown to exert protective effects during ischemic brain injury (Smith-Swintosky et al., 1994), and overexpression of APP in transgenic mice was shown to confer resistance to kainate-induced and chronic forms of excitotoxicity (Mucke et al., 1996; Masliah et al., 1997; Steinbach et al., 1998). As for *in vitro* approaches, however, the relevant cellular and molecular players involved in APP-mediated neuroprotection await identification.

## (2) Physiological role of A $\beta$

A $\beta$  has been extensively studied in the context of its association with plaques in AD brains, but A $\beta$  also exists in normal individuals without any known pathology. A $\beta$  is released in lower amounts in normal brains throughout life during synaptic activity and seems to be beneficial for normal brain synaptic functions (Puzzo et al., 2008). The *in vivo* cerebral concentration of A $\beta$  has been estimated to be in the picomolar range (Cirrito et al., 2003) for both the cerebrospinal fluid and plasma of healthy rodents throughout life (Seubert et al., 1992) and growing evidence indicates that this peptide might have physiologic functions. Indeed, APP, and  $\beta$ - and  $\gamma$ -secretases have been shown to be involved in synaptic plasticity and memory (Dawson et al., 1999; Phinney et al., 1999; Seabrook et al., 1999; Saura et al., 2004; Laird et al., 2005; Ma et al., 2007; Puzzo et al., 2008; Wang et al., 2008; Puzzo et al., 2011; Puzzo et al., 2012) and A $\beta$  itself, at picomolar concentrations, is likely to play a neurotrophic and neuroprotective role (Yankner et al., 1990; Plant et al., 2003; Lopez-Toledano and Shelanski, 2004; Giuffrida et al., 2010). In a recent and elegant study, Puzzo et al. (2012) have clearly shown that A $\beta$  effects on synaptic plasticity and memory are dependent on the concentration of A $\beta$  used (Puzzo et al., 2012). Indeed, these authors show that A $\beta$  acts on hippocampal LTP and reference memory drawing biphasic dose-response curves. This phenomenon, characterized by low-dose stimulation and high-dose inhibition raises important issues on the use of A $\beta$  level-reducing agents in AD. Interestingly, Cirrito et al. (2008) have demonstrated that synaptic transmission induces an increase of A $\beta$  generation and release, and Kamenetz et al. (2003) suggested that endogenous A $\beta$  might regulate synaptic plasticity with a feedback mechanism (Kamenetz et al., 2003; Cirrito et al., 2008). Thus cognitive deficit in AD may be the consequence of a homeostatic imbalance of

the physiologic feedback mechanism exerted by A $\beta$  on synaptic activity. It has been also suggested that A $\beta$  may interfere with the insulin receptor pathway. Indeed, it is well-known that neuronal synapses and astrocytes of memory-processing brain regions possess insulin receptors (IRs) (Lee et al., 2005a) which, when activated by insulin, facilitate synaptic plasticity in the normal brain (Ding et al., 2006). It has been shown that the monomeric A $\beta$  is able to activate the IGF-1/IRs by locally produced IGF-1 and that A $\beta$  may also directly bind to IGF-1/IRs, as already shown for A $\beta$  oligomers (Xie et al., 2002; Townsend et al., 2007).

Altogether, these findings clearly involve the monomeric form of A $\beta$  in several brain functions. Because most of the physiological effects of A $\beta$  have been described only for the monomeric form, it has been suggested that A $\beta$  aggregation might contribute to AD pathology through a "loss-of-function" process.

**Remark:** The majority of AD mice are based on transgenic overexpression of APP in combinations with different familial AD-associated mutations in APP or PS1 genes. This overexpression of APP generates elevated A $\beta$  levels to mimic the A $\beta$  amyloidosis of AD brains, but also automatically generates increased levels of APP fragments including sAPP, CTF $\alpha$ , CTF $\beta$ , and AICD. As we have just described above, some of these fragments have biological functions and may cause several potential A $\beta$ -independent artifacts associated with APP overexpression that may influence our data interpretation.

***g) Imbalance of A $\beta$  production and clearance leads to Alzheimer's disease***

In the interstitial fluid of normal brains, A $\beta$  concentration is due to a dynamic equilibrium between its rate of **production from the APP** via an **influx into the brain across the blood-brain barrier (BBB)** mainly via receptors for advanced glycation end products (RAGE) (Deane et al., 2003), and by its rapid **clearance across the BBB** via low-density lipoprotein receptor related protein-1 (LRP1) (Shibata et al., 2000; Deane et al., 2004b; Deane et al., 2004a) and **enzymatic degradation within the brain** (Selkoe, 2001a). In humans, A $\beta$  is estimated to have a physiological production rate of 7.6% per hour and a clearance rate of 8.3% per hour (Bateman et al., 2006). The various mechanisms of removal provide greater A $\beta$  clearance than production, thus limiting its accumulation. Interestingly,

human data provide evidence that accumulation in sporadic AD results from impaired clearance rather than increase production of A $\beta$  (Mawuenyega et al., 2010). Using an in vivo labeling technique, Mawuenyega et al. found that the clearance rate of A $\beta_{1-42}$  in AD patients was reduced to 5.3% per hour in comparison to 7.6% per hour in healthy controls. Likewise, the A $\beta_{1-40}$  clearance rate was reduced to 5.2% per hour compared to 7.0% per hour in controls. This finding emphasizes the importance of A $\beta$  clearance in sporadic AD.

#### (1) Enzymatic degradation of A $\beta$

The proteolytic degradation of A $\beta$  is a major route of clearance. The number of enzymes capable of proteolytic degradation of A $\beta$  discovered to date is rapidly growing and currently the family of amyloid-degrading enzymes includes more than 20 members both membrane-bound and soluble, and of differing cellular and subcellular locations (Marr and Spencer, 2010; Nalivaeva et al., 2012; Grimm et al., 2013; Pacheco-Quinto and Eckman, 2013). Intriguingly, several of the main amyloid-degrading enzymes are members of the M13 peptidase family (neprilysin (NEP), NEP2 and endothelin converting enzymes). Another metallopeptidase from the M16 family, the insulin-degrading enzyme, also plays a significant role in A $\beta$  degradation in the brain (Leissring et al., 2003; Qiu and Folstein, 2006). All these enzymes have been mostly studied in recent years with regard to their role in AD pathology or as therapeutic targets. It has been shown that NEP mRNA and protein expression levels are reduced with age and in AD subjects (Reilly, 2001; Yasojima et al., 2001; Iwata et al., 2002; Apelt et al., 2003; Caccamo et al., 2005; Maruyama et al., 2005; Wang et al., 2005; Wang et al., 2010). Using brain tissue from various brain regions of non-impaired, mild-cognitive impairment and AD subjects, NEP2 (which displays slower and weaker A $\beta_{1-40}$  degrading properties when compared to neprilysin) mRNA expression levels in the mid-temporal gyrus were found to be lowered in women with mild-cognitive impairment compared to non-impaired women (Huang et al., 2012).

Interestingly, most of the factors which predispose to the development of sporadic AD (such as hypoxia, ischaemia, stroke, stress, diabetes, trauma and neuroinflammation) were shown to affect the levels of expression and activity of amyloid-degrading enzymes. As an example, the experimental *four-vessel occlusion ischemia model* in rats resulted in down-

regulation of NEP and endothelin converting enzymes as well as up-regulation of APP and BACE both in the cortex and hippocampus (Nalivaeva et al., 2004).

## (2) Clearance across blood-brain barrier

Thus, continuous removal of A $\beta$  species from the brain by transport across the BBB and/or metabolism is essential to prevent their potentially neurotoxic accumulations in brain tissue (Zlokovic et al., 2000). The mammalian brain is separated from blood by the BBB localized in the brain capillaries and pia-subarachnoid membranes and the blood cerebrospinal fluid barrier situated in the choroid plexi. The physical sites of these barriers are tight junctions between brain endothelial cells and epithelial cells, respectively (Redzic and Segal, 2004; Smith et al., 2004; Zlokovic, 2008). There are no effective barriers to diffusion of molecules between brain interstitial fluid and cerebrospinal fluid. While the vascular barriers restrict the transport of polar solutes, rapid transport of essential hydrophobic nutrients, such as glucose and amino acids, and peptides and proteins, involves specific transporter systems and/or receptor-mediated transport, respectively (Zlokovic, 2004).

However, the major clearance transport mechanism of free monomeric A $\beta$  is transcytosis across the BBB which is mediated mainly by the cell surface LRP1 receptor localized predominantly on the abluminal side of the cerebral endothelium (Shibata et al., 2000; Deane et al., 2004b). LRP1 is the main receptor for A $\beta$  transport across the BBB in the direction of brain to blood. Since **LRP1 expression in brain capillary endothelium decreases during normal aging** (in rodents and nonhuman primates), and in AD (Shibata et al., 2000; Bading et al., 2002; Deane et al., 2004b), **it reduces A $\beta$  clearance and promote A $\beta$  cerebrovascular** and brain focal accumulations. Binding of A $\beta$  to LRP1 at the abluminal side of the BBB in vivo initiates a rapid A $\beta$  clearance via transcytosis across the BBB into blood in mice and rats (Shibata et al., 2000; Deane et al., 2004b; Cirrito et al., 2005; Bell et al., 2007; Sagare et al., 2007; Deane et al., 2008). In human plasma, a soluble form of LRP1 (sLRP1) is a major binding protein for circulating A $\beta$  (Sagare et al., 2007). Human sLRP1 sequesters about 70 to 90 % of plasma A $\beta$  (Sagare et al., 2007). In contrast, circulating A $\beta$ , excepted when it is sequestered by sLRP1, may also enter into the brain mainly by a specific receptor-mediated transport mechanism that is dependent on RAGE expression on the luminal surface of brain vessels (Mackic et al., 1998; Deane et al., 2003).

A number of transport proteins, such as albumin, apolipoprotein E (apoE), apolipoprotein J (apoJ), transthyretin (TTR), and  $\alpha$ 2-macroglobulin ( $\alpha$ 2M) binds A $\beta$  (Biere et al., 1996; Narita et al., 1997) and influences its clearance, metabolism and aggregation (Zlokovic et al., 1996; Deane et al., 2008). As an example, apoE disrupts the rapid LRP1-dependent clearance of free monomeric A $\beta$  across the mouse BBB, in an isoform specific manner (apoE4>apoE3 or apoE2), by redirecting A $\beta$  transport from LRP1 to very low density lipoprotein receptor (VLDLR) which internalizes A $\beta$ -apoE complexes at a slower rate than LRP1 (Deane et al., 2008).

Thus, RAGE and LRP1 on the BBB and sLRP1 in plasma play a key role in controlling brain interstitial fluid A $\beta$  concentration. In plasma, sLRP1 sequesters A $\beta$  and maintains the peripheral 'sink' action which maintains continuous A $\beta$  clearance from the brain. **In AD, RAGE levels at the BBB are increased and LRP1 levels at the BBB and the capacity of sLRP1 binding of peripheral A $\beta$  are reduced, favoring A $\beta$  accumulation in the brain.**

#### ***h) Regulation of A $\beta$ production***

There is considerable and still growing clinical and epidemiological evidence that genetic or environmental factors (metals, traumatism brain injury, stroke...) can trigger an imbalance of A $\beta$  production significantly increasing the risk to develop AD.

- (1) Genetic factors: Mutations associated to amyloid cascade

Most of the mutations responsible for familial AD have been shown to either increase production of total A $\beta$  load or specifically A $\beta$ <sub>1-42</sub>, the longer and more aggregable A $\beta$  species (Checler, 1995, 1999). Mutations on APP or presenilins are responsible for most of the genetic cases underlying familial forms of AD.

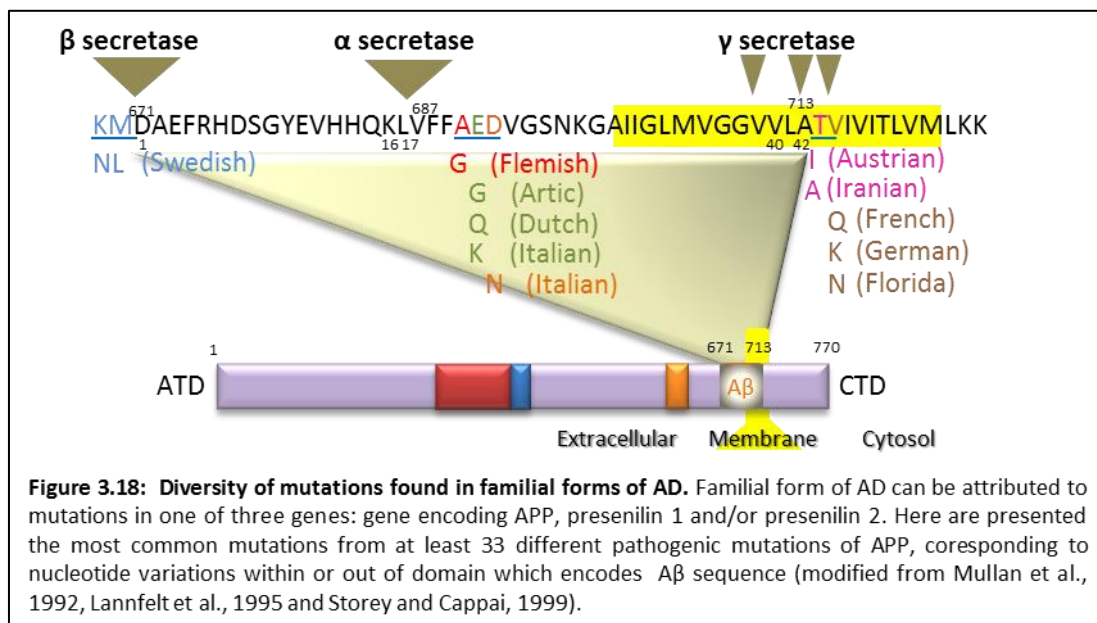
- (2) APP gene mutation

The first mutations associated with familial forms of AD were discovered in the APP gene. Most of these pathogenic mutations, in the segment which encodes amyloid beta, can be classified into three groups. The first group of mutations is located near to the cleavage



site of  $\gamma$ -secretase, with a shift in amino acid residues between 713 and 717 (exon 17) of the amyloid precursor protein gene. The representative example for this missense mutation is the London mutation where an amino acid substitution, causing a Val to Ile (Val717Ile) replacement, occurs (Talarico et al., 2010). All these mutations are pathogenic and lead to development of AD, causing an increased relative ratio  $A\beta_{1-42}/A\beta_{1-40}$  (De Jonghe et al., 2001). The second group of mutations leads to a change in the APP of the  $\beta$ -secretase site of action. The only known mutation so far is the Swedish double mutations in residues 670 and 671, and it is associated with an AD and cerebral amyloid angiopathy (CAA) pathology (Storey and Cappai, 1999). The mutation is at the amino-terminus of  $A\beta$  and predicts lysine-to-asparagine and methionine-to-leucine substitutions at exon 16 (Mullan et al., 1992; Lannfelt et al., 1995).

The third group of mutations is located at the domain encoding  $A\beta$  part of APP and affects one of the residues in the region 692 to 694. The examples of these mutations are Dutch variant (E693Q) and Flemish variant (A692G) (Storey and Cappai, 1999) (Figure 3.18).



### (3) PS1 gene mutation

Mutations in the PS1 gene on **chromosome 14** (14q24.3, 75kb, 12 exons) result in the most severe form of the disease with autosomal dominant inheritance, complete penetrance until age of 60, and onset occurring by the fourth decade of life or earlier. The first mutation was reported by Schellenberg et al. in 1992 and since then more than 180,

mostly missense and rarely small deletions or insertions mutations, have been found (Schellenberg et al., 1992). In a study conducted on 31 families with autosomal dominant early onset AD, frequency of PS1 mutation was the highest (Janssen et al., 2003). Work on the molecular phenotype of AD has demonstrated that these mutations result in the overproduction of the form of the A $\beta$  ending in amino acid 42 (Poorkaj et al., 1998). Also, some mutations result in a faster rates of neurofibrillary tangles formation, such as M139V, I43F, G209V, but not E9 (Gomez-Isla et al., 1999).

#### (4) PS2 gene mutation

Presenilin 2, homolog of PS1, primary expressed in neuronal cells in the cerebral cortex and in the hippocampus is encoded by a gene located on **chromosome 1** (1q42.13) (McMillan et al., 1996). First mutations were reported by Rogaev et al. and Levy Lohad et al. in 1995 and since then less than 15 additional mutations have been found ([www.molgen.ua.ac.be/ADMutations/](http://www.molgen.ua.ac.be/ADMutations/)) (Levy-Lahad et al., 1995; Rogaev et al., 1995). Mutations in this gene lead to development of autosomal dominant AD with onset age of 40-75, with possible complete penetrance.

#### (5) Environmental factors

##### (a) *Metals*

Metals are ubiquitous contaminants, particularly arsenic-cadmium-lead, which are among the major toxic agents found in environment and act as important risk factors for AD (Dosunmu et al., 2007). These environmental pollutants exert their actions by modifying the amyloidogenic pathway. Arsenic is reported to affect expression and processing of APP in neuronal cells (Zarazua et al., 2011). Cadmium reduces non-amyloidogenic processing of APP (Li et al., 2012), and lead co-localizes within and facilitates amyloid plaque formation in transgenic mouse brains (Gu et al., 2012). Neonatal lead exposure is also reported to promote late-onset amyloidosis in aging rodents and monkeys (Basha et al., 2005b; Basha et al., 2005a; Wu et al., 2008).

##### (b) *Traumatic brain injury*

Traumatic brain injuries (TBIs) have been identified as an environmental risk factor for AD (Heyman et al., 1984; Mortimer et al., 1991; Guo et al., 2000; Plassman et al., 2000;

Uryu et al., 2002; Fleminger et al., 2003). Indeed, among patients suffering from head injury, 30% display A $\beta$  deposition within 7 days (Roberts et al., 1991; Roses and Saunders, 1995). In addition, study pointed out the transient upregulation of  $\beta$ -secretase BACE-1 expression 24-72 h after TBI, accompanied by an elevation of its activity detected 48 h after onset (Blasko et al., 2004). The molecular mechanisms of TBI-induced upregulation of  $\beta$ -secretase could be related to an increased expression of neuronal Kunitz protease inhibitory domain (KPI) in the APP protein which inhibits the  $\alpha$ -secretase candidate tumor necrosis factor-alpha converting enzyme (TACE) (Lesne et al., 2005). The consequence of this KPI-dependent inhibition is a shift from  $\alpha$ -secretase to  $\beta$ -secretase-mediated APP processing and then to an increase in A $\beta$  production.

### *i) A $\beta$ aggregation*

The biochemistry of A $\beta$  proteins has been a fascinating area of research because of its contribution to our understanding of the pathogenetic mechanisms of AD. The A $\beta$  sequence is amphipathic, with the first 28 amino acid residues containing both hydrophobic and hydrophilic groups, whereas the remaining residues are apolar and uncharged (Soreghan et al., 1994). A $\beta$  is a member of the class of proteins known as **intrinsically disordered proteins** (IDPs) – proteins that lack a stable tertiary structure in physiological conditions. IDPs are known to have many binding partners due to their flexibility of conformation (Uversky and Dunker, 2013). As a consequence of extracellular accumulation of A $\beta$ , monomeric units interact non-covalently to form oligomers (Bemporad and Chiti, 2012) and finally fibers and plaques. Many different oligomeric forms of A $\beta$  can exist simultaneously in a dynamic equilibrium and have been referred to as amorphous aggregates, micelles, protofibrils, prefibrillar aggregates, ADDLs, A $\beta$ \*56, globulomers, amylospheroids, “tA $\beta$ ” (toxic soluble A $\beta$ ), “paranuclei,” and annular protofibrils (Glabe, 2008). A $\beta$  aggregates ranging from dimers up to particles of one million Dalton or greater have been reported *in vitro* (Glabe, 2008). Electron microscopy and atomic force microscopy have also identified spherical particles of ~3-10 nm that appear at early times of incubation and disappear as mature fibrils appear (Harper et al., 1997; Anguiano et al., 2002; Lashuel et al., 2002). These spherical oligomers appear to represent intermediates in the pathway of fibril formation because they are transiently observed at intermediate times of incubation during fibril formation. The conformation of A $\beta$  fibril is better known and studies have

showed that residues 18-26 and 31-42 form the  $\beta$  strands. Salt bridges between Asp23 and Lys28 stabilize the turn region connecting the two  $\beta$  strands (Nelson and Eisenberg, 2006; Finder and Glockshuber, 2007).

Although all forms of A $\beta$  have a great propensity towards aggregation in aqueous solution, the major form found in plaques is A $\beta$ <sub>1-42</sub>. The relative abundance of A $\beta$ <sub>1-42</sub> with respect to A $\beta$ <sub>1-40</sub> reflects the fact that even a small elongation of the stretch of hydrophobic residues in the carboxyl-terminal region increases dramatically the tendency of this peptide to aggregate (Jarrett et al., 1993).

Remark: **This lack of a native fold results in A $\beta$  occupying a large conformational space. This space is highly dependent on environment (Kodali and Wetzel, 2007), making the oligomer states very sensitive to perturbation by the procedures used for analysis.** As a good example, studies have reported that formation of dimers and trimers could be induced by sodium dodecyl sulfate (SDS). As a consequence, a number of reports have pointed out that one of the most common methods for defining oligomer distributions, SDS-PAGE, may produce misleading results (Bitan and Teplow, 2005; Watt et al., 2013).

## 2. Amyloidopathy

### a) *Amyloid Oligomers versus amyloid plaques*

Originally, the A $\beta$  hypothesis of AD was based on the assumption that cognitive impairment was the consequence of A $\beta$  fibril deposition in senile plaques. However, alterations in synaptic connectivity and/or strength as well as cognitive deficits are observed in transgenic mouse models of AD before the appearance of thioflavin S positive plaques (Hsia et al., 1999; Mucke et al., 2000; Jacobsen et al., 2006). These observations in animals fit nicely with evidence that memory and cognitive deficits in MCI and AD patients correlate far better with cortical A $\beta$  levels than with plaque numbers (Naslund et al., 2000) and correlate best with the soluble pool of cortical A $\beta$ , which includes soluble oligomers (Kuo et al., 1996; Lue et al., 1999; McLean et al., 1999). Another piece of compelling evidence of soluble oligomers being a causative factor in AD comes from studies in which A $\beta$  was injected directly into various brain areas of animals, primarily the hippocampus. In these studies, microinjections of the synthetic, natural, and human AD-derived A $\beta$ <sub>40</sub> impaired working

memory in a manner consistent with this type of memory deficit in AD patients (Cleary et al., 2005; Lesne et al., 2006; Shankar et al., 2008). Considerable advances have been made in the past 15 years with increased recognition that soluble A $\beta$  oligomers are the major neurotoxins which cause synaptotoxicity in the early phases of the pathology. The final aggregation into plaques seems to represent an irreversible process (Lim et al., 2007; Bernstein et al., 2009), but these plaques might serve as reservoirs for the oligomers (Lesne et al., 2006).

## **b) Cellular target(s) of A $\beta$**

### **(1) Membranes**

A $\beta$ , as well as other proteins associated with and involved in its synthesis and clearance (PS1, BACE1, APP, and  $\beta$ CTF) are identified to be present within the lipid rafts. Lipid rafts are dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the bilayer, mostly at the plasma membrane. They have an important role in membrane signaling (Simons and Ikonen, 1997). GM1 ganglioside, one of the major components of these domains, or cholesterol was shown to interact with A $\beta$  *in vitro*, *in vivo* and in human CSF (Hong et al., 2014). This accumulation of A $\beta$  may cause the toxicity by interfering with signal transduction (Morishima-Kawashima and Ihara, 1998). It was recently found that oligomeric forms of A $\beta$  bound strongly to GM1 ganglioside, and by blocking the sialic acid residue on GM1, decrease oligomer-mediated LTP impairment in mouse hippocampal slices (Hong et al., 2014).

Finally, oligomers may also form ion-permeable pores in cell membranes, causing unregulated calcium entry and thereby leading to synaptic toxicity (Lashuel and Lansbury, 2006).

### **(2) Receptors**

Beside the possibility to attach directly to plasma membrane, it is proposed that A $\beta$  binds to the different receptors at the membrane and thus exert its toxicity. These A $\beta$  receptors include NMDAR, RAGE,  $\alpha$ 7 nicotinic acetylcholine receptor ( $\alpha$ 7 nAChR), cellular prion protein, ephrin type B receptor 2, immunoglobulin G Fc gamma receptor IIb, and paired immunoglobulin-like receptor B (Kam et al., 2014).

### (3) Intracellular accumulation

Intraneuronal accumulation of A $\beta$  has been observed in the early stages of AD (McGeer et al., 1992). However, in older individuals in late stages of AD, when abundant plaques are present in the brain, intraneuronal accumulation of A $\beta$  is less evident (Gouras et al., 2000). In several mouse models of AD, intraneuronal accumulation of oligomeric A $\beta$  has been found (Kumar-Singh et al., 2000; Shie et al., 2003) and has led to altered synapse structure in the hippocampus (Price et al., 2014; Zou et al., 2015). However, the presence and/or the function of the different forms of A $\beta$  are still debated.

#### ***c) Pathophysiology***

##### (1) Amyloid $\beta$ and synaptic transmission

When within a physiological range, small increases in A $\beta$  primarily facilitate presynaptic functions, resulting in synaptic potentiation. A $\beta$  could act as a positive regulator at the presynaptic level. For example, relatively small increases in endogenous A $\beta$  levels enhance the release probability of synaptic vesicles and increase neuronal activity in neuronal culture (Abramov et al., 2009). Another study reported that application of low concentrations of synthetic A $\beta_{1-42}$  (picomolar range) markedly potentiate synaptic transmission, whereas higher concentrations of A $\beta_{1-42}$  (low nanomolar range) cause the expected synaptic depression (Puzzo et al., 2008). In this study, the potentiating effect of A $\beta$  was found to be dependent on  $\alpha 7$ -nAChR activation. A $\beta$  may directly act on presynaptic  $\alpha 7$ -nAChR (Dineley et al., 2002) through increasing of presynaptic Ca<sup>2+</sup> levels. A positive modulatory effect of A $\beta$  on synaptic transmission is further supported indirectly by the finding that abnormally low levels of A $\beta$  in mice deficient for APP (Seabrook et al., 1999), PS1 (Saura et al., 2004), or BACE1 (Laird et al., 2005) are associated with synaptic transmission deficits.

In contrast, abnormal A $\beta$  levels and assembly forms may indirectly cause synaptic depression and loss (Kamenetz et al., 2003; Hsieh et al., 2006; Shankar et al., 2007). This is consistent with the fact that A $\beta$  impairs LTP (Walsh et al., 2002a; Shankar et al., 2008; Li et al., 2011) and enhances LTD (Kim et al., 2001; Hsieh et al., 2006; Li et al., 2009). It is interesting to note that multiple lines of evidence suggest that there is an early, pre-plaque

phase when learning and memory deficits are not detected in AD transgenic mice, but when LTP is already impaired (Oddo et al., 2003a; Jacobsen et al., 2006).

Although the mechanisms underlying A $\beta$ -facilitated LTD have not yet been fully elucidated, they may involve receptor internalization (Snyder et al., 2005; Hsieh et al., 2006) or desensitization (Liu et al., 2004a) and subsequent collapse of dendritic spines (Snyder et al., 2005; Hsieh et al., 2006). A $\beta$ -dependent effects on synaptic function may be mediated by postsynaptic activation of  $\alpha$ 7-nAChR (Snyder et al., 2005), activation of extra-NMDARs (Shankar et al., 2007; Li et al., 2009), and downstream effects on calcineurin/STEP/cofilin, p38 MAPK, and GSK-3 $\beta$  signaling pathways (Wang et al., 2004; Shankar et al., 2007; Li et al., 2009; Tackenberg and Brandt, 2009).

## (2) Synaptic loss and amyloid $\beta$

Studies that have used immunohistochemistry and other techniques to evaluate possible synaptic changes in different brain regions have reported AD-associated changes in specific synaptic proteins in the hippocampus (Harigaya et al., 1996), frontal, temporal and parietal lobes (Reddy et al., 2005). These changes include both presynaptic (synaptophysin, synaptobrevin, SNAP 25, synaptotagmin, syntaxin, rab3a, synapsin I) and postsynaptic proteins (PSD-95, drebrin). The overall picture from these studies is that the loss of synapses and/or synaptic proteins is extremely widespread in AD and not limited to a specific region of the cortex (Scheff et al., 2014). There was a significant correlation between the synaptic density and the MMSE (assessment of cognitive function), suggesting that loss of synaptic connectivity in the frontal cortex is associated with lower MMSE scores (Scheff and Price, 2003). Synaptic volume density in lamina III was significantly lower in the AD patient's brain at autopsy compared to controls, showing a significant loss of synapses in the magnitude of 35% (Scheff and Price, 2003). Synapse loss appears to be an early event in the disease process. These changes have been also observed in several models of AD mice (Hsia et al., 1999) and can be reproduced *in vitro* by adding A $\beta$  oligomers (Shankar et al., 2007). To date, A $\beta$  fibrils, A $\beta$  oligomers, or the intracellular accumulation of A $\beta$ , have been found to alter the function and structure of dendritic spines by distinct mechanisms (Dorostkar et al., 2015).

Although A $\beta$  have been found to alter the function and structure of dendritic spines, tau hyperphosphorylation (Dickstein et al., 2010; Hoffmann et al., 2013) and microglia

activation (D'Amelio et al., 2011; Tong et al., 2012; Pozueta et al., 2013; Erturk et al., 2014), which are thought to be consequences of amyloidosis in AD, may also contribute to spine loss.

### (3) Hyperphosphorylation of tau

*In vitro* experiments using various cell types, ranging from neuronal cell lines to hippocampal organotypic cultures, have showed A $\beta$ -induced tau alterations. These alterations include increased tau phosphorylation and cytoplasmic and dendritic tau translocation, often linked to neurodegeneration. A growing list of different animal models of AD has reproducibly and robustly recapitulated A $\beta$ -induced tau pathology (Stancu et al., 2014). Although initial mouse models expressing mutant APP or mutant APP/PS1 without overexpression of tau did not display neurofibrillary tangles or robust tau aggregation in the brain, subtle changes on endogenous mouse tau, like tau hyperphosphorylation, have been described. And finally, dynamic biomarker analyses as indicators of key pathological features in human patients with AD have addressed the temporal evolution of these processes in relation to each other and to disease progression. Earliest changes appeared in CSF A $\beta$ <sub>1-42</sub>, closely followed by amyloid PET imaging with an important lag period before the first symptoms. Alterations in CSF tau (t-Tau and p-Tau) appear later in the disease process and precede cognitive decline and brain atrophy (Jack et al., 2010). This sequence is completely in line with the amyloid cascade hypothesis and the initiator role of A $\beta$  in tau pathology. Together **these data could position A $\beta$  as an “initiator” and tau as an “executor” of AD.**

## F. Role of NMDARs in A $\beta$ pathology

### 1. Role of NMDARs in A $\beta$ -mediated synaptic dysfunction

Accumulating evidence supports a role of NMDARs in the etiology of AD, via A $\beta$ -mediated effects on synaptic dysfunction (Malinow, 2012). Effects of A $\beta$  on synaptic depression (inhibition of LTP or potentiation of LTD) have been consistently reported in different APP transgenic mouse models and in different experiments using extracellular application of different forms of A $\beta$  on hippocampal slices (Selkoe, 2002; Palop and Mucke, 2010; Ittner and Gotz, 2011; Malinow, 2012; Mucke and Selkoe, 2012). Several studies have indicated downstream effects of A $\beta$  on NMDAR function (Snyder et al., 2005; Dewachter et



al., 2009; Malinow, 2012). A $\beta$  oligomers have been demonstrated to bind to synaptic sites in primary neuronal cultures, co-localizing, but not completely overlapping, with NMDARs (Snyder et al., 2005; Dewachter et al., 2009). Hence, A $\beta$  peptides have been proposed to indirectly interact with NMDARs, potentially through the EphB2 receptor, a tyrosine kinase receptor known to regulate NMDARs or other receptors (Cisse et al., 2011). Different reports have further demonstrated that prolonged A $\beta$  incubation promotes endocytosis of synaptic NMDARs, particularly the GluN2B-containing, resulting in depression of NMDA evoked currents and reduced CREB signaling required for long-term memory (Snyder et al., 2005; Dewachter et al., 2009; Malinow, 2012; Kessels et al., 2013). Moreover, several mechanisms have been invoked to explain the opposite effect of A $\beta$  on LTP and LTD, but recent studies have suggested that the effect of A $\beta$  on LTD could involve extrasynaptic NMDARs. Indeed, soluble A $\beta$  may block neuronal glutamate uptake at synapses, leading to increased glutamate levels at the synaptic cleft (Li et al., 2009). A resultant would be a glutamate spillover and activation of extrasynaptic NMDARs, enriched in GluN2B-containing NMDARs, which play a major role in LTD induction (Liu et al., 2004) and have also been shown to mediate the inhibition of LTP induced by soluble A $\beta$  (Li et al., 2011). More recently, the Malinow's group has suggested that a switch in NMDAR composition from GluN2B to GluN2A (Kessels et al., 2013) could explain the synaptic depression induced by A $\beta$ .

## 2. NMDARs can influence A $\beta$ production

Stimulation of synaptic NMDARs has been shown to increase levels of  $\alpha$ APPs and phosphorylation of extracellular signal regulated kinase (ERK), and to decrease production of A $\beta$ <sub>1-40</sub>. These effects were blocked by NMDAR antagonists (Hoey et al., 2009). However, chronic over stimulation of NMDARs with agonists can lead to an increase of production of A $\beta$  (Lesne et al., 2005). These opposing results can be explained by different localization and composition of NMDARs. In this sense, a recent study demonstrated that only the activation of the extrasynaptic NMDARs increased the A $\beta$  production, by causing the shift from the APP695 to the KPI-containing APP isoform with high amyloidogenic properties. In contrast, the activation of synaptic NMDARs did not trigger amyloidogenesis (Bordji et al., 2010).

### 3. Role of extrasynaptic NMDARs in AD

Besides altering synaptic plasticity, excitotoxicity caused by glutamate has been proposed to be a mechanism contributing to progressive neuronal loss in AD (Pomara et al., 1992; Harkany et al., 2000). Indeed, soluble A $\beta$  oligomers have been recently found to interrupt glutamate reuptake (Matos et al., 2008; Li et al., 2009) and to increase extracellular glutamate levels, which have led to the recruitment of extrasynaptic receptors. Several recent studies have demonstrated that increased extrasynaptic NMDAR activity plays a key role in the pathogenic mechanisms of Huntington's disease (Okamoto et al., 2009; Milnerwood et al., 2010) and of glutamate excitotoxicity (Stanika et al., 2009). These findings further support a scenario in which synaptic NMDARs activate cellular survival pathways, whereas extrasynaptic NMDARs tend to activate pathways that contribute to cell death (Hardingham et al., 2002; Papadia and Hardingham, 2007). Interestingly, memantine, a NMDAR antagonist used to treat more advanced AD patients, has been shown to prevent A $\beta$ -mediated inhibition of LTP in rodents *in vivo* (Klyubin et al., 2011). This compound mainly target extrasynaptic NMDARs (Milnerwood et al., 2010; Xia et al., 2010). Taken together, we can hypothesize that overactivation of extrasynaptic NMDARs in the hippocampus may be an early step in the pathogenesis of AD, leading to the amnesic symptoms associated with AD and ultimately, neuronal death.

**Remark:** More recently studies have also shown that tau controlled the location of Src-family tyrosine kinase Fyn, which increases NMDAR phosphorylation.

### 4. Memantine, a well-tolerated NMDAR antagonist

#### a) Properties

Memantine, derivative of amantadine (an anti-influenza agent), is an uncompetitive, voltage-dependent, NMDAR antagonist with fast on and off kinetics and with a moderate receptor binding affinity. It blocks the NMDAR-associated ion channel with higher affinity, but lower voltage-dependency, than the natural blocker-magnesium. Memantine, also known as an open channel blocker, can enter the channel only after its opening and then blocks current flow. The fact that memantine and Mg<sup>2+</sup> bind at the same place in the receptor's pore is supported by the Mg<sup>2+</sup> reduction of memantine blockade. The M2 loop

region of GluN1 subunit of NMDARs is a critical determinant of divalent cation permeability and  $Mg^{2+}$  blockade. In particular, asparagine residues in this region are part of  $Mg^{2+}$  binding site and contribute to the selectivity filter of the channel (Kashiwagi et al., 2002; Johnson and Kotermanski, 2006). Compared with the physiological blockade by external  $Mg^{2+}$ , memantine exhibits a slower unblocking rate, moderate voltage dependence and slightly higher affinity (Danysz and Parsons, 2003).

### ***b) Problematic***

Memantine is an **open NMDAR-channel blocker**. At first glance, it seems illogical that this mechanism of action leads to the reported beneficial effects of the drug, knowing that the role of NMDARs is extremely important for the processes of LTP, synaptic plasticity and learning (Parsons et al., 2007). However, this antagonist is more effective in blocking excessive activity because statistically, when there are more channels that are open, there are more channels available to be blocked (Chen and Lipton, 2006). During pathological activation of the NMDARs, memantine blocks excessive calcium entry through the channel induced by chronic overstimulation of the NMDARs (Chen and Lipton, 1997). Thus, memantine leads to a higher degree of channel blockade in the presence of excessive levels of glutamate, and little blockade at relatively lower levels, for example, during physiological neurotransmission (Chen and Lipton, 2006), **which could explained the good tolerance of this NMDAR antagonist**. More recently, studies have shown that memantine could specifically block extrasynaptic NMDARs, mainly involved in glutamatergic overactivation processes, without interfering with synaptic NMDARs, which serves physiological functions such as learning and memory.

### ***c) Memantine and Alzheimer's disease***

The efficacy of memantine in the management of patients with AD, vascular dementia, and mixed dementia was assessed in a Cochrane meta-analysis including 12 randomized controlled trials (McShane et al., 2006). The meta-analysis showed that memantine was superior to placebo in benefiting cognitive function for mild-to-moderate AD and moderate-to-severe AD (McShane et al., 2006). More recently, an updated meta-analysis of randomized placebo-controlled trials testing memantine monotherapy for patients with AD and including 2433 patients confirmed that memantine monotherapy

improved cognition, behavior, activities of daily living, global function, and stage of dementia and was well-tolerated by AD patients (Matsunaga et al., 2015).

### **G. Models of Alzheimer's disease**

Many of the therapeutic or preventive interventions that have been used so far have shown minimal or no effect on cognition and improvement of everyday life in AD patients. To better understand and characterize the onset and development of disease, more than 100 mouse models have been developed (<http://www.alzforum.org>). These various transgenic models are aimed at reproducing mostly the familial (early onset) AD, which only represents 5% of all AD cases and occurs due to mutations in the APP, presenilin1 and/or presenilin 2 genes. As reviewed in Zaks and Ashe (2010), there are still no AD transgenic mice carrying one mutation that can produce all the hallmarks and symptoms of human AD (Zaks and Ashe, 2010). Although none of these transgenic mouse models completely recapitulates human development and progression of AD, they have been invaluable in understanding and pinpointing the molecular mechanisms underlying the pathology. Rather than fully covering all aspects of the disease, these models can for instance provide an insight into different aspects of cognitive impairment related to AD (Webster et al., 2014). Most of the models develop amyloidopathy without tauopathy, but they differ in the beginning of symptom onset, speed of development and progression, as well as in the severity of the disease.

One of the most extensively used transgenic model to study amyloidogenesis, is the Tg2576 model which carries a single mutation of the APP encoding gene resulting in a 14-fold increase in  $A\beta_{1-42}/A\beta_{1-40}$  ratio and in subsequent amyloid plaques deposition, but without neurofibrillary tangles and neuronal loss (Hsiao et al., 1996), and with a variation in the onset of cognitive impairment between studies (Westerman et al., 2002). Impairment in working memory and contextual fear conditioning has been reported starting at six months of age although other studies have reported normal cognition at this age with progressive impairment at 12 months of age or even later (Kim et al., 2015; Puri et al., 2015).

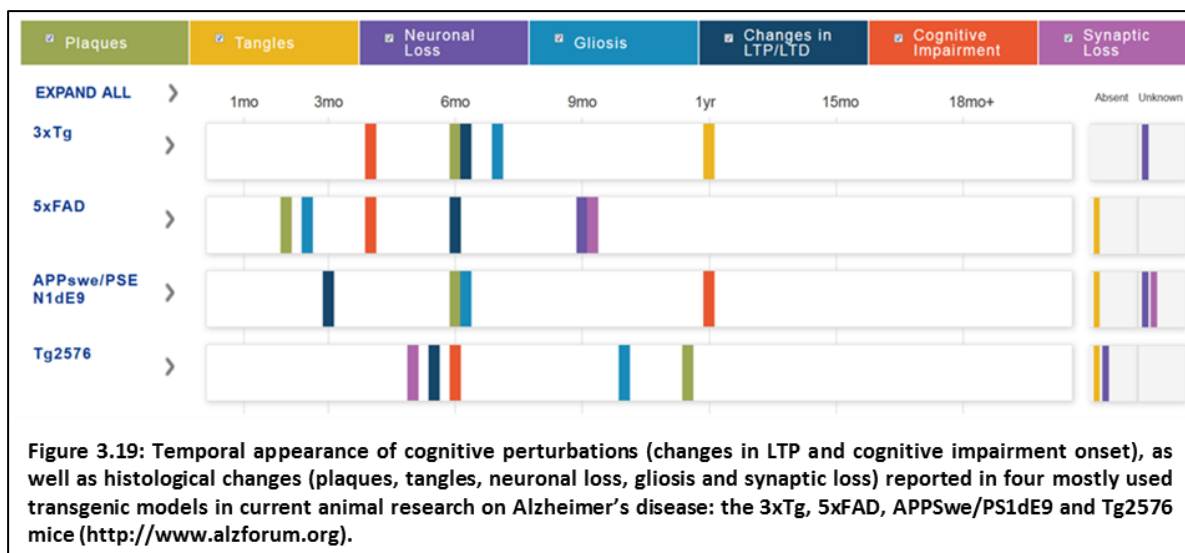
Double transgenic mouse models co-expressing mutated APP together with another mutation, such as PS1, develop amyloid pathology much earlier and show behavioural deficits earlier in life (Rowan et al., 2003; Eriksen and Janus, 2007) than mice expressing only

the sole APP mutation. Overexpression of the APP with the Swedish mutation (APP<sup>swe</sup>) and PS1 with deletion in exon 9 (PS1<sup>dE9</sup>) leads to overproduction of APP and PS1 splice variants with concomitant increase in parenchymal A $\beta$  load and in an increased A $\beta_{1-42}$ /A $\beta_{1-40}$  ratio. It has been reported that these APP<sup>swe</sup>/PS1<sup>dE9</sup> double transgenic mice develop the first A $\beta$  plaques at 4 months of age, but the onset of the cognitive decline occurs at an older age (Savonenko et al., 2005). By 12 months of age, the mice develop deficits in spatial tasks measuring spatial navigation and reference learning. APP<sup>swe</sup>/PS1<sup>dE9</sup> mice still do not exhibit neuronal death, but they display a variety of other clinically relevant AD-like symptoms, which makes this model suitable for studying synaptic dysfunction related changes (Malm et al., 2011). This is the model that we used in our experimental studies.

The 5xFAD model expresses five familial AD mutations (Swedish, Florida and London mutations in the APP gene and two PS1 related mutations) that contribute to the rapid development of severe amyloid pathology, by increasing A $\beta_{1-42}$  production. 5xFAD mice exhibit intraneuronal A $\beta_{1-42}$  accumulation at a really early age, 1.5 months, amyloid deposition at 2 months, and synaptic degeneration and memory deficits by 4 months of age (Oakley et al., 2006; Eimer and Vassar, 2013), without however signs of the presence of NFTs.

All these models, despite the presence of human APP and/or PS1 mutations, do not lead to development of NFTs. Therefore a novel model has been introduced (3xTg) which carries three mutations in the APP, tau and PS1 genes. Accordingly these mutant mice develop both amyloid plaques and NFTs. Cognitive changes in this model occur before the onset of plaque and tangle accumulations. Extracellular A $\beta$  deposits appear by six months of age and further progress with aging, while changes in tau occur significantly later around the age of 12 to 15 months (Oddo et al., 2003b; Billings et al., 2005).

In Figure 3.19 is shown the phenotype comparison of representative and mostly used AD transgenic models in current animal research on AD (<http://www.alzforum.org>).



The development of these transgenic models allowed a major breakthrough in the understanding of AD mechanisms but it was initially based on the hypothesis that cognitive deficits, and in particular memory deficits, were linked to development of plaques. Considerable advances have been made in the past decade with increased recognition that only soluble oligomeric forms of amyloid  $\beta$  ( $A\beta$ ) are the major neurotoxins which cause synaptic dysfunction in the early phases of the pathology (Shankar and Walsh, 2009). The first experimental direct evidence of the role of  $A\beta$  in generating memory deficits has been demonstrated in 2005 by using intracerebral administration of amyloid peptides. In an elegant set of experiments, Cleary and colleagues (2005) have clearly shown, for the first time, that  $A\beta$ , including trimers and dimers, are necessary and sufficient to disrupt learned behavior in a manner that is rapid, potent and transient (Cleary et al., 2005). The memory profile observed following  $A\beta$  intracerebral injections were indicative of a pathophysiological action similar to that seen with amnesic drugs such as scopolamine (Cleary et al., 2005). Thus, intracerebral injections of  $A\beta$  have been developed as an alternative to transgenic animals to study the effects of an increase of soluble amyloid species in the brain without presence of any plaques. Several studies consistently showed that microinjections of the synthetic, natural, and human AD-derived  $A\beta$  impaired working memory (Cleary et al., 2005; Lesne et al., 2006; Shankar et al., 2008) and that soluble  $A\beta$  dimers were necessary and sufficient to disrupt normal learning and memory functions.

However, as other models, the intracerebral  $A\beta$  injection model presents some differences when compared to the human disease. For example, an acute intracerebral

injection does not reflect the progressive character of AD due to the relatively fast onset of action of the A $\beta$ o solution. Acute injections may however adequately mimic synaptic dysfunction (impairment of plasticity) resulting from excess of A $\beta$ . Moreover, an intra-structure injection can produce a concentration gradient of the amyloid species from the site of injection to remote brain regions and concentration in some structures may not reach levels seen the disease. This injection may also produce tissue damage at the injection site. For this reason, intracerebroventricular administrations have been most often preferred to intra-structure microinjections considering the volume of CSF in ventricles and the fact that the needle tip is not directly in the tissue. In turn, this may also ease A $\beta$  distribution to remote structures and minimize tissue reactions such as inflammation at the injection site (Chambon et al., 2011). The group of Luc Buée has recently validated a model in which several intrahippocampal injections of oligomeric forms of A $\beta$  were successful in inducing the main characteristics of A $\beta$  pathology (synaptic loss, hyperphosphorylation of tau, cognitive deficit and even neuronal death) that only a few transgenic mice were able to reproduce (Brouillette et al., 2012). Much faster than other transgenic animal models in reproducing cognitive deficit, this model avoids also the variability of behavioral data related to the genetic background of transgenic mice. **However, one of the major weaknesses of this model is related to conditions of A $\beta$  preparation which could lead to crucial changes in the A $\beta$  aggregation state.** Indeed, many factors are critical when preparing an amyloid solution (temperature, solvent, pH) and may shorten or extend the lag phase which precedes the aggregation of amyloid peptides. Thus, a mix of different amyloid species can be found in the final solution, i.e. monomer, oligomer, fibril, and what is critical (and is a source of variability) is the proportion of these different forms.

These findings highlight the interest of using the intracerebral A $\beta$ o model in conjunction with specific transgenic mouse models. While transgenic mice can be useful to identify new pathological process, the intracerebral model can offer a rigorous control over the time course of the A $\beta$  pathology, making it possible to dissect efficiently or identify new cellular and molecular mechanisms underlying the A $\beta$  pathology.

## **OBJECTIVES OF THE THESIS**



Cognitive functions are fundamental to human life as illustrated by the distress of AD patients when memories progressively and irremediably disappear. Elucidation of the mechanisms underlying memory processes in normal and pathological conditions could lead to the development of novel therapeutic targets or strategies aimed at alleviating memory loss. It is now well known that AD does not only affect different forms of memories (dependent of the pathological stage) but also interferes with an array of complex memory processes ranging from encoding, consolidation and retrieval.

Because of the diversity of memory forms and processes affected in AD, we have chosen to characterize further the cognitive signature of the double transgenic APP<sup>swe</sup>/PS1<sup>dE9</sup> mice in order to pinpoint specific cognitive deficits in this widely used animal model of AD. To this end, we developed an innovative test battery composed of seven behavioural paradigms tailored to targeting hippocampal-dependent working, reference and episodic-like memory and specific memory processes (acquisition, consolidation, retrieval and flexibility). Because environmental enrichment has been proposed to provide a cognitive reserve that could be beneficial for preventing memory deficits associated with AD, we also examined the impact of early and late exposure to EE on the cognitive signature of our APP<sup>swe</sup>/PS1<sup>dE9</sup> mice. These results appear in Part A of the result section.

As mentioned in the general introduction, since the initial findings that NMDARs play important roles in cellular models of learning and memory as well as neurotoxicity, abnormal functions of these receptors have been considered a potential mechanism in the pathophysiology underlying AD. Indeed, since A $\beta$ s bind preferentially to the postsynaptic density of neuronal excitatory synapses, where the scaffolding proteins organize the subunit composition (GluN2A and GluN2B) of NMDARs and their subcellular location (synaptic versus extrasynaptic), we raised the hypothesis that the early synaptic accumulation of A $\beta$  could alter the scaffolding protein organization and consequently, the NMDAR subcellular location, which would in turn impair the plasticity-related events triggered by learning and memory processes. To test this possibility, we adopted an integrative strategy which favored vertical levels of analyses (from phenotype to molecular events). We specifically designed a set of interrelated correlative approaches using the double transgenic mouse model of AD (APP<sup>swe</sup>/PS1<sup>dE9</sup>) and their age-matched wild-type controls combined with invasive

approaches (manipulation of NMDAR activation) in normal mice. These results are presented in part Part B of the result section.

Although the hippocampus encodes information during periods of active wakefulness, it has become clear that memory traces are reactivated within hippocampal-cortical networks during offline periods, such as sleep, facilitating their progressive embedding and long-term storage in the cortex. This memory reactivation process is supported, at least in part, by high-frequency bursts during slow-wave sleep called sharp wave ripples (SWRs), which could also be dependent of NMDAR activation. Whereas the functional implication of these SWRs in memory processes in the normal brain is rather well documented, surprisingly, the functional contribution of SWRs to the spatial memory impairments associated with AD remains poorly understood.

To provide novel insights into this unresolved issue, we developed and validated a new version of the Y-maze offering a higher spatial cognitive demand and chose the model of intracerebroventricular injections of soluble oligomeric forms of A $\beta$  in adult mice. This choice over a typical transgenic mouse model was motivated by the observation that cognitive deficits of AD patients are correlated to soluble A $\beta$  levels rather than plaque-development *per se*. In addition, this model enables rigorous control over the time course of AD symptomatology, a property which is compatible with the recordings of A $\beta$ -induced changes in SWRs occurrence rate in the hippocampus of cognitively-challenged mice submitted to a spatial discrimination. Results of these experiments appear in the last part, Part C, of the result section.

Overall, by adopting an integrative strategy which combined behavioral, molecular and electrophysiological characterizations of learning and memory deficits in mouse models of AD, we were able to identify two new disruptive mechanisms underlying the spatial memory deficits typically associated with AD: 1) an A $\beta$ -induced destabilization of the synaptic organization and increase of the extrasynaptic pool of GluN2B-containing NMDARs in the hippocampus, a reorganization which translated into impaired memory functions, and 2) an A $\beta$  -induced abolishment in the post-encoding and post-recognition peaks in SWR occurrence normally observed in control mice, indicating impaired hippocampal processing of spatial information and an inability to form and stabilize long-term memories. These two

mechanisms are further discussed in light of the existing knowledge on the AD symptomatology and a putative model of abnormal forgetting in AD incorporating our findings, and describing how amyloidogenesis impacts the organization of recent and remote spatial memory, is proposed.

## IV. MATERIALS AND METHODS

## **A. Ethical considerations**

Experimental procedures complied with official European Guidelines for the care and use of laboratory animals (directive 2010/63/UE) and were approved by the ethical committee of the University of Bordeaux (protocol A50120159).

## **B. Animals**

The double transgenic APP<sub>swe</sub>/PS1dE9 mice were obtained by crossing the single APP<sub>swe</sub> line (male, Tg 2576) and PS1dE9 transgenic mice (female). The background strain for APP<sub>swe</sub> was C57BL/6xSJL (Taconic Inc, Germantown, NY, USA), and that for PS1dE9 was C57BL/6 (Jackson Laboratory, Bar Harbor, ME, USA). Male mice, with Swedish APP mutation, carried human APP<sub>695</sub>, containing the double mutation K670N and M671L (APP770 numbering), the isoform derived from a large Swedish family with early-onset AD. These mice develop significant amyloid plaques and display memory deficits. Mice co-express human presenilin 1 (PS1) carrying the exon-9-deleted variant (PSEN1dE9) associated with familial AD. Both were under the control of the mouse prion protein (PrP) promoter, directing transgene expression predominantly to CNS neurons.

The age of the mice at the beginning of the experiment ranged from 9 to 10 months. Male APP<sub>swe</sub>/PS1dE9 and WT littermates were used to characterize the cognitive signature of male APP<sub>swe</sub>/PS1dE9 mice, to pinpoint specific cognitive deficits and to study the alteration of NMDAR function. Female APP<sub>swe</sub>/PS1dE9 and WT littermates were used to evaluate the sensitivity of our APP<sub>swe</sub>/PS1dE9 mouse model to the effects of environmental enrichment (EE). All mice were heterozygous with respect to the transgene, and genotypes were confirmed by polymerase chain reaction (PCR) of tail biopsy. Adult C57BL/6J (Janvier) mice were also used for the intracerebral A $\beta$  model which consisted in infusing A $\beta$  oligomers into the lateral ventricles. Two weeks prior to the beginning of behavioral experiments, the mice were housed in individual cages. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986.

## **C. Environmental enrichment**

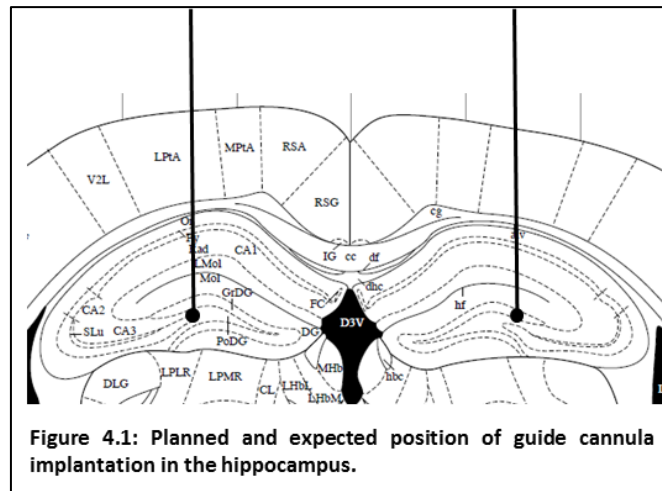
Female APP<sub>swe</sub>/PS1dE9 and WT littermates were exposed to EE during 10 weeks in early adulthood, at 2 months of age (before amyloidogenesis occurred), and later in

adulthood, at 7 months of age (when the pathology was already present). The effect of EE on the cognitive profile on enriched and non-enriched (home cage) mice was tested at 9 months. EE consisted of a large box (100×70×40cm) containing various toys, wooden blocks, climbing platforms, plastic tubes, small houses, but no running wheel. The toys, food and water were rearranged and renewed every three days to stimulate the animals' exploratory behavior. Mice were exposed to EE by groups of 10 to 15. Animals assigned to the non-enriched (NE) group remained in standard laboratory cages by groups of 5 for the entire duration of the experiment. In both housing conditions, animals had free access to food and water. After completion of the 10-week period of enrichment, mice were returned to their home cage where they remained until the age of 9 months. Their behavior was then tested.

#### **D. Intracerebral injections in freely moving animal**

##### **1. Guide cannula implantations into the hippocampus**

Adult C56BL/6J male mice (3-4 months) from Janvier were implanted bilaterally with guide cannula under deep general anesthesia. Each mouse was anesthetized with vetflurane (induction, 4%; maintenance, 1.5-2%) and secured in a Kopf stereotaxic frame. To avoid the dry eyes, gel was applied as needed during the whole surgery. Body temperature was controlled and maintained at a stable 37 °C. The scalp was then cut and retracted to expose the skull. Craniotomies were made directly above the target regions, and the dura was cut to expose the cortex. The holes in the skull were drilled before lowering the stainless steel guide cannulas at the following coordinates: posterior -2 mm, mediolateral -/+1.4 mm, and dorsoventral -1 mm (Figure 4.1). After placing, the guide cannulas were secured by dental cement. Topical analgesic was applied immediately following the surgical procedure and as needed thereafter.



## 2. Intracerebral injections into freely moving mice

All infusions took place in a preparation room separate from the behavioral testing area. Animals were restrained gently by the experimenter throughout the infusion process. The infusion cannulas, which extended 1 mm beyond the tip of the guide cannulas, were inserted into the guides. Bilateral infusions were conducted simultaneously using two Hamilton syringes which were connected to the infusion cannulas by propylene tubing. The syringes were driven by a Harvard Apparatus precision syringe pump, which delivered 0.5 $\mu$ l of DL-TBOA or DL-TBOA+ifenprodil or aCSF or lidocaine to each hippocampus over a 2 min period. The infusion cannulas were left in place for an additional 1.5 min to allow the diffusion of the drugs. The infusion cannulas were then removed. For all experiments, and before behavioral testing, mice were familiarized with all aspects of the procedure described above except that the injection cannulas were not connected to the infusion pump. This was done to reduce stress levels that could interfere with subsequent memory testing.

## E. Intracerebroventricular infusions of A $\beta$

### 1. Preparation of A $\beta$

The peptide used in our studies was obtained from Bachem. Prior to resuspension, each vial was allowed to equilibrate to room temperature for 30 min to avoid condensation upon opening the vial. The first step in resuspending, the lyophilized peptide was treatment in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma). Each vial of peptide was diluted in 100% HFIP to 1 mM. The clear solution containing the dissolved peptide was then aliquoted in

microcentrifuge tubes. The HFIP was allowed to evaporate in the fume hood. Immediately prior to use, the HFIP-treated aliquots were carefully and completely resuspended to 2mM in anhydrous dimethyl sulfoxide (Sigma) by pipette mixing followed by bath sonication for 15 min. Then, the sample was dissolved in 95  $\mu$ l of ice-cold PBS immediately vortexed for 30s, and incubated at 4°C for 24h. Final concentration obtained for the A $\beta$ o was 100  $\mu$ M (store at -20°C).

## 2. Intracerebroventricular infusion

To model certain aspects of AD, we used an intracerebroventricular (ICV) injection of A $\beta$ o as previously described by Balducci et al. (2010)(Balducci et al., 2010). The oligomeric forms of A $\beta$ , or vehicle alone-artificial cerebrospinal fluid (aCSF), were administered ICV to anesthetized mice in a total volume of 4  $\mu$ l. The mice were anesthetized by 4% Vet-Flurane, placed in a stereotaxic frame (Kopf, USA) and the injection site was appropriately prepared. A small incision was made, the skull was exposed and cleaned and a small hole was drilled using a dental drill. The A $\beta$ o, or vehicle solution was injected into the right lateral ventricle using a Hamilton syringe connected to a cannula at a rate of 0.5  $\mu$ l/min controlled by a syringe pump (Harvard Apparatus, Holliston, MA, USA). The following stereotaxic coordinates were used: 1 mm posterior to bregma, 1.3 mm lateral to sagittal suture, and 2 mm dorsoventral. The incision was closed using Vet-Bond, and the mouse was placed on an isothermal pad at 37°C and continuously observed following surgery until recovery. Topical analgesia was applied immediately following the surgical procedure and as needed thereafter.

## F. Electrophysiology recording

### 1. Surgery

After habituation to the vivarium conditions, male C57BL/6J mice (3-4 months) underwent stereotaxic surgery under deep isoflurane anesthesia. To model certain aspects of AD, we used an intracerebroventricular injection of A $\beta$ o as previously described. Just after, bilateral electrodes consisting of an insulated tungsten wire (diameter 35  $\mu$ m, California Fine Wires) were implanted into the CA1 region of the hippocampus (AP: -2.0 mm, L:  $\pm$ 1.5 mm, V: -1.05 mm). Reference and ground electrodes were implanted into the



cerebellum. The electromyogram (EMG) electrode was inserted into the neck muscles. All electrodes were welded to a 6-pin connector attached to the skull with dental acrylic cement.

## 2. Recording

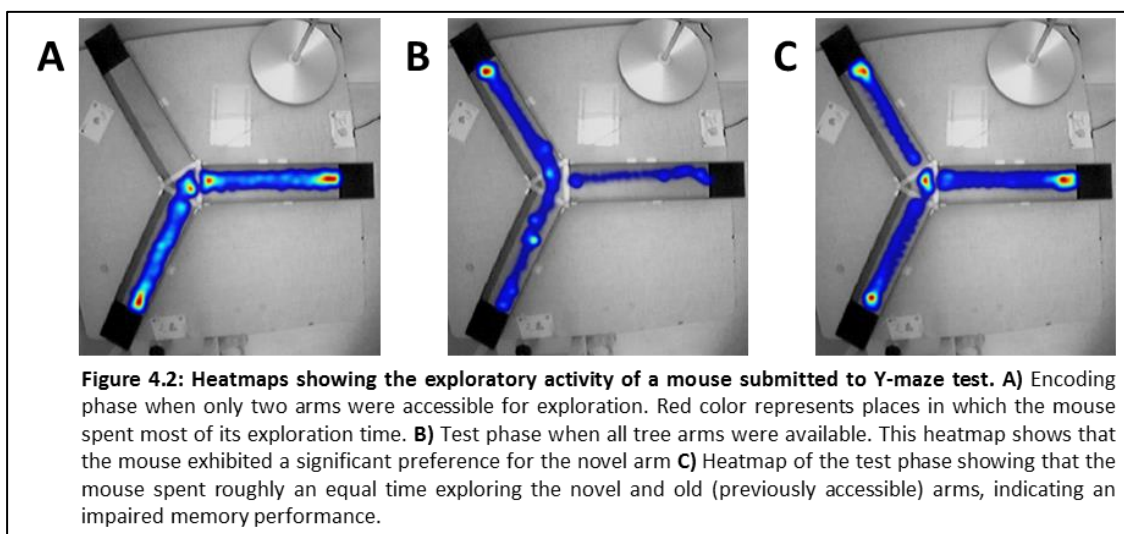
Daily recordings were performed from 9 am to 4 pm in a closed opaque and dimly illuminated box which could host the animal's home cage. The mouse head connector was linked to amplifiers by a soft cable allowing free motions of the animal. Behavior was tracked with a video camera. Electroencephalograms and EMG signals were amplified by a differential home-made AC amplifier, digitized at 32 kHz with 16-bit resolution using CED Power 1401 converter and Spike2 software (Cambridge Electronic Design), and stored on a PC for off-line analysis. Data processing was performed by using Neuroscope (<http://neuroscope.sourceforge.net/>) and Matlab. Sonic Vizualizer was used to display and analyze the spectrograms. Local field potential (LFP) recordings were down-sampled to 1250 Hz and EMG was band-pass filtered to 250-350 Hz. Power spectra of delta (1-5 Hz) and theta (5-10 Hz) frequency band were calculated continuously. Brain states corresponding to awake, REM and slow wave sleep (SWS) states were manually scored by the experimenter using EMG, spectrogram delta/theta ratios as cues as well as video-recording. SWS states were identified as episodes of immobility (tonic EMG) and high delta power. SWS bouts separated by less than 3 seconds were merged. REM states were identified as episodes of high theta and low delta power accompanied by atonic neck EMG recording. SWRs were detected by means of the FMA Toolbox (<http://matoolbox.sourceforge.net>) only during periods classified as SWS. After bandpass-filtering LFP data at 100-250 Hz, SWRs were identified by thresholding the normalized squared signal (NSS) if its envelope exceeded 2 standard deviations (SD) and peak exceeded 5 SD. The time points when the NSS crossed 2 SD were considered as onset and offset of a SWR. Episodes lasting longer than 100 ms were excluded from the analysis whereas episodes separated by less than 30 ms were merged. The occurrence rate was expressed as number of SWRs per 1 sec of SWS. Power was calculated as maximum of NSS within a ripple. Time bins containing less than 5 min of a total duration of SWS were excluded from analysis of SWRs dynamics.

## G. Behavioral testing

APPswe/PS1dE9 mice and non-transgenic WT littermates were submitted to a cognitive test battery to assess different memory processes.

### 1. Classical Y-maze

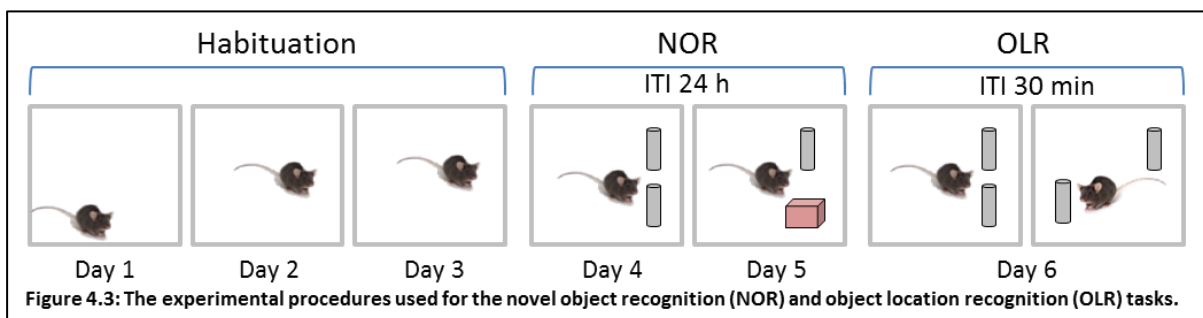
The Y-maze apparatus was made of gray Plexiglas and consisted of three arms at a 120° angle from each other. Each arm was 8 cm×30 cm×15 cm (width × length × height). The three identical arms were randomly designated: “start arm”, in which the mouse started to explore (always open), “novel arm”, which was blocked during the 1st trial, but open during the 2nd trial, and “other arm” (always open). The testing procedure consisted of two trials separated by an inter-trial interval (ITI) to assess spatial recognition memory. The first trial (encoding) had 5-min duration and allowed the mouse to explore only two arms (start arm and other arm) of the maze, with the third arm (novel arm) blocked. After 10 min ITI (Wang et al., 2006), the second trial (recognition) was conducted during which all three arms were accessible and novelty versus familiarity was analyzed by comparing time spent in all three arms. Video recordings were later analyzed and the number of entries and time spent in each arm were analyzed. Discriminating the novel arm from the two familiar arms during the first 2 min of exploration was considered as an index of spatial recognition memory (Akwa et al., 2001). Memory performance was thus expressed as the percentage of time spent in novel arm ( $((\text{seconds in novel arm})/(\text{seconds in previously visited arms} + \text{seconds in novel arm}) \times 100)$ ). The mice were tracked by using the Noldus videotracking system (Figure 4.2).



## 2. Novel object recognition (NOR) and object location recognition (OLR) tasks

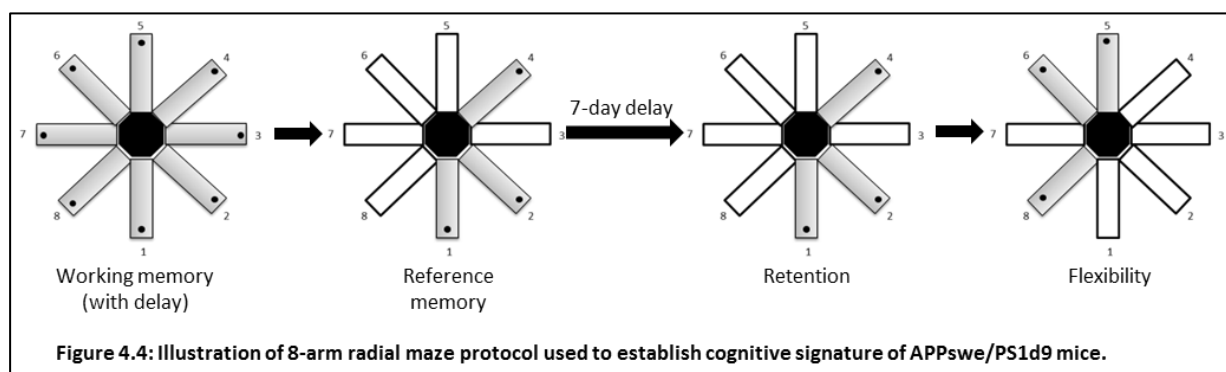
NOR test assesses the ability of mice to recognize a novel object in the environment (Ennaceur and Aggleton, 1997). For three consecutive days, the mice were individually habituated to a 40cm×40cm square-shaped open field with clear Plexiglas walls for 10 min. On the fourth day, the mice were allowed to explore two same objects until the sum of time spent exploring each object equaled 60 seconds, this in order to ensure that the amount of information encoding (and presumably the strength of the memory trace) was similar across tested mice. Twenty four hours later, the mouse was then submitted to an object novelty recognition test in which a novel object replaced one of the familiar objects (Figure 4.3). The mouse could explore this environment until the sum of time spent exploring each object equaled 30 sec.

In the OLR task, the mouse's ability to recognize that an object it had encountered before has changed location was assessed. In the acquisition phase, mice were exposed to similar objects, which were placed in adjacent corners of the arena. The animals were allowed to explore both objects until the sum of time spent exploring each object equaled 60 sec. After a delay of 30 min, the test phase began in which one object was moved in the corner opposite to its original position (Figure 4.3). The position of the moved object was counterbalanced between mice. All objects and the arena were thoroughly cleaned with 70% ethanol between trials and animals to remove odors. Memory was expressed as a discrimination index, that is  $(\text{seconds on novel} - \text{seconds on familiar}) / (\text{seconds on novel} + \text{seconds on familiar}) \times 100$ . Animals with no memory impairment spent longer time investigating the novel object, giving a higher discrimination index. The mice were tracked by using the Noldus videotracking system.



### 3. 8-arm radial maze (8-ARM)

Spatial memory testing was conducted using an 8-arm radial maze. Each arm was 62 cm long and 12 cm wide and radiated from a central platform (32 cm in diameter). Different memory forms were assessed: working memory, working memory with delays, reference memory, retention and flexibility (Figure 4.4). The mice were tracked by using the Imetronic system (Pessac, France).



Two weeks before testing, mice were housed one per cage with the *ad libidum* access to food and water. Mice were manipulated daily and their free feeding weights were taken over 3 consecutive days. Food deprivation, until mice reached 85–90% of their free feeding weights, was performed and kept during the entire period of testing. For testing in radial maze, sweet milk pellets were used as rewards. Mice were weighed after the test, and the adjusted amount of regular food was given to maintain a stable level of food deprivation.

Mice were submitted to two daily sessions of habituation during which they could freely explore the maze. On the first habituation day (H1), each of the 8 arms of the maze was baited with a sweet milk pellet placed at the end of each arm and also in the middle to encourage exploration. All doors of the maze were open and the mouse could freely explore the maze. After visiting each arm, doors closed to prevent visiting an arm twice. During the second habituation day (H2), rewards were placed only at the end of each arm and not visible from the central platform. Door stayed open during the whole trial, so repeating visits (repetition) of an arm were possible. The trial ended when the mouse visited all 8 arms. During H1 and H2, mice had only 1 trial per day.

#### 4. **Working memory**

After 2 days of habituation, food-deprived mice were tested for working memory. All 8 arms were baited. Between each entry in an arm, doors were closed during 4 seconds to keep the mouse in the central platform and to prevent it from using clockwise or counterclockwise exploration strategy that would prevent the mice from using its memory. Number of arm entries before first repetition and total number of repeated arms were used as an index of performance. Mice had 2-4 consecutive daily trials for 4 days. Each trial was terminated when mouse collected pellets from all 8 arms.

#### 5. **Working memory with delay**

The same rules, as for the working memory procedure, were applied only the time spent on the central platform after 4 correct entries was increased from 4 seconds to 30s or 180s during one trial.

#### 6. **Reference memory**

The same 3 arms out of 8 were always baited with the milk pellets. Each mouse had a different set of baited arms, but the positions of the baited arms were always 45°-90°-225° (for instance arms: 1-2-4, 2-3-5, 3-4-6, etc.). Mice had 6 consecutive daily trials for 10 days. Each trial, separated by one min, was terminated when the mouse collected the pellets from the three baited arms. Visit to a non-baited arm was scored as a reference memory error.

#### 7. **Retention**

Animals were tested for retention 7 days after the end of acquisition. The same 3 arms were baited for each mouse. One single retention session made of 6 trials was administered to each mouse. The same procedure as for acquisition was applied.

#### 8. **Flexibility**

During the two days following retention, mice were first submitted to retraining in the radial maze with the same arms baited (6 trials/day) in order to ensure a similar level of task mastery across groups. Flexibility testing could then be evaluated. The flexibility test

consisted in 6 trials per day during 6 days, but with 3 new arms baited (reversal session). New arms were selected based on a 180° rotation (for instance arms: 1-2-4→5-6-8). Number of entries in old arms versus number of entries in newly baited arms during the first 8 entries of a trial was recorded and used as an index of memory flexibility

### **9. Y-maze in 8-arm radial maze**

To increase the spatial cognitive demand of our regular Y-maze discrimination procedure, we adapted it to the 8-arm radial maze in which only three arms were used to form a Y-shape (90°-135°-135° between the arms). Each arm was 62 cm long and 12 cm wide and radiated from a central platform (32 cm in diameter). Behavioral procedure was composed of the exploration (encoding) phase and the recognition phase, which were separated by various ITI: 10min, 2h, 4h or 24h. During the encoding trial, one of the three available arms was closed. The mouse was positioned on the central platform of the maze and allowed to explore the two available arms during 10 min. During the recognition trial, the exploration time of each of the three arms was automatically recorded during a 5 min period with the entrance into an arm scored when the first half of the body was inside the arm. Mice normally tend to explore the previously blocked arm (novel arm) of the maze more often than the previously accessible (familiar) ones. Because this behavioral paradigm relies on novelty seeking, the recognition trial should not be repeated and animals were used only once. Discriminating the novel arm from the two familiar arms was thus considered as an index of spatial recognition memory. Memory performance was expressed as the percentage of time spent in novel arm  $((\text{seconds in novel arm}) / (\text{seconds in previously visited arms} + \text{seconds in novel arm})) \times 100$ . Time spent on the central platform of the maze was excluded from the calculation of performance. Chance level was set at 33% of the exploration time.

### **H. Verification of guide cannula position**

Cannula placements were examined after behavioral testing to ensure correct implantation. Mice were deeply anesthetized by lethal intraperitoneal injection of pentobarbital and the whole brain was collected and frozen at -80°C. A cryostat was used to generate coronal sections (30 µm thick) proximal to guide and cannula tracts and mounted on PLUS slides (Fisher Scientific) until dried. Cannula placements were verified under a light

microscope. Since injection sites were as expected within the dorsal hippocampus for all mice, no mice were excluded from the data analyses.

## I. **Euthanasia and brain extraction**

For brain biochemical analysis, mice were euthanized by lethal intraperitoneal injection of pentobarbital, and decapitated. Brains were extracted, and hippocampi and brain cortex were separated in ice cold solution of PBS and kept at -80°C until further analyses. The hippocampi of each animal were frozen separately to be treated independently for fractionation or total protein extraction.

## J. **Biochemical analysis**

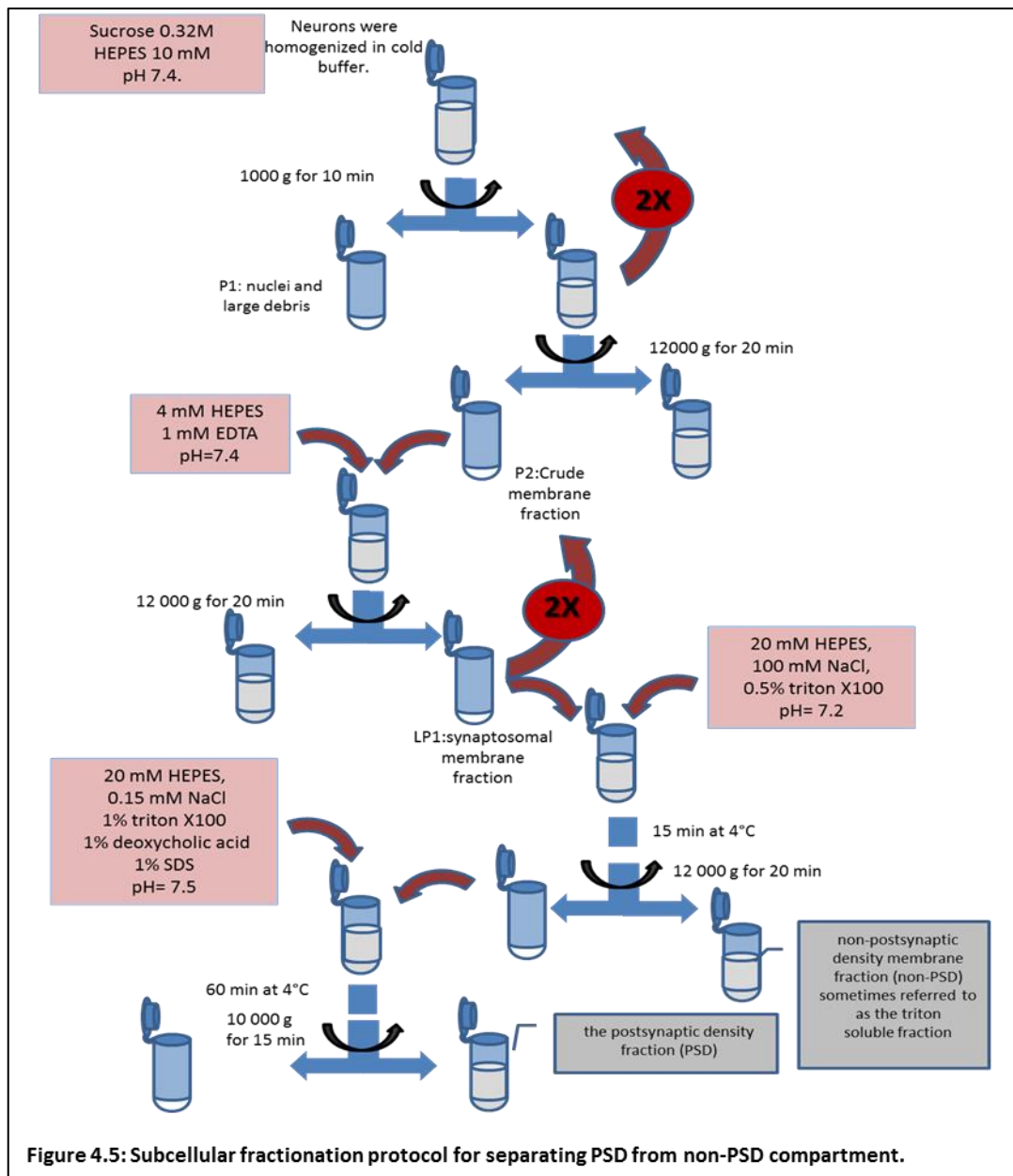
### 1. **Total protein extraction**

Hippocampi from transgenic and non-transgenic WT littermates were dissociated by using a 18G needle in a 100 µL solution containing 20 mM HEPES, 0.15 mM NaCl, 1 % triton X100, 1 % deoxycholic acid, 1 % SDS, pH 7.5 (buffer IV). After Incubation for 1 hour at 4°C, the samples were next centrifuged at 10 000 g during 15 min. Supernatant was isolated and used as a total protein extract.

### 2. **Subcellular fractionation**

Subcellular fractionation was performed as described in Figure 4.5. Tissues were re-suspended in cold buffer containing 0.32 M sucrose supplemented with 10 mM HEPES, pH 7.4 (Buffer I). Homogenates were cleared one time at 1 000 g for 10 min, and then the supernatants were concentrated at 12 000 g for 20 min. Thus, the crude membrane fraction was rinsed during 20 min at 12 000 g with a buffer containing 4 mM HEPES, 1 mM EDTA, pH 7.4 (Buffer II). This fraction was then incubated in 20 mM HEPES, 100 mM NaCl, 0.5 % triton X100, pH 7.2 (Buffer III) during 15 min and centrifuged 20 min at 12000 g. The supernatant was considered as the non-postsynaptic density (non-PSD) membrane fraction. The pellet was solubilized for 1 hour in 20 mM HEPES, 0.15 mM NaCl, 1 % triton X100, 1 % deoxycholic acid, 1 % SDS, pH 7.5 (Buffer IV) and then centrifuged at 10 000 g during 15 min. The supernatant obtained contained the postsynaptic density (PSD) fraction. All buffers were

supplemented with protease and phosphatase inhibitors (Sigma, L'Isle d'Abeau, France), and protein concentration was measured using the BCA kit (Pierce).



### 3. Western blotting

#### a) Protocol

Protein amounts of brain homogenates were determined by the Bradford's protein assay and normalized to 5-20 µg of protein per sample. Twelve percent NuPAGE Bis-Tris polyacrylamide gels (Invitrogen, France) were used to run the electrophoresis (80 min, 120V). After size separation within the gel, proteins were transferred to a polyvinylidene



difluoride (PVDF) membrane (Polyscreen® membrane, Perkin Elmer, France). Membranes were blocked with a solution containing 0.1% Tween 20 and 200 mM Tris buffered solution (TTBS) complemented with 5 % non-fat dry milk during 30 min and incubated with primary antibody, at 4°C overnight under gentle agitation. Incubation with the secondary fluorescent-conjugated antibody was performed during 1 h at room temperature. After 3 washes with TTBS and one with PBS the membrane was scanned using a Licor Aeries automated infrared imaging system according to manufacturer's instructions. Quantification based on band intensity was done with the software provided with the imaging system.

Because of the low amount of sample, the membrane was reused two times. Antibody deshybridation was performed by incubating the membrane in a stripping buffer (0.2 M glycine, 0.1 % SDS, 1 % Tween 20, adjusted pH 2.2) during 5-10 min. After several washes with TTBS, the membrane was scanned to control the efficiency of the stripping buffer. After this control, the membrane was incubated with TTBS containing 5 % non-fat dry milk and then incubated with another antibody in the same conditions as described before.

#### ***b) Antibodies***

Different primary antibodies were used: 4G8 (Covance, 1 µg/ml), Anti-Synaptophysin (Millipore, 1 µg/ml), anti-PSD95 (Millipore, 2 µg/ml), anti-GluA1 (Millipore, 0.1 µg/ml), anti-GluN2A (Millipore, 0.1 µg/ml), anti-GluN2B (Millipore, 1 µg/ml), anti-Actin (Sigma Aldrich, 0.8 µg/ml), anti-CamKII $\alpha$ 1 (Santa Cruz, 1 µg/ml) and anti-NR1 (Santa Cruz, 1 µg/ml). The adequate secondary antibodies were used: Goat anti-Rabbit (IR Dye® 800CW, LI-COR) or Goat anti-Mouse (IR Dye® 680 RD, LI-COR).

#### **4. Determination of brain A $\beta$ <sub>1-42</sub> by ELISA assay**

Human A $\beta$ <sub>1-42</sub> was quantified in the same amount of total, PSD or non PSD hippocampal extract from APP<sup>swe</sup>/PS1dE9 mice and WT littermates by ELISA (BetaMark Covance, USA). According to the manufacturer, this kit specifically detects soluble forms of A $\beta$ <sub>1-42</sub> with negligible cross-reactivity to A $\beta$ <sub>1-40</sub>. A $\beta$  concentration in samples was read in a 96-well plate against a standard curve (0-250 pg/ml). Chemiluminescence signals were detected using a microplate reader.

## K. **Statistical analyses**

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (<http://www.graphpad.com>). N represented the number of independent animals that were analyzed. Data sets were tested for normal distribution with Kolmogorov–Smirnov tests. In cases of normal distribution, t-test, one-way and two-way ANOVAs were performed to test for possible differences among groups. Bonferroni’s test was used for post hoc comparisons. For non-normal distributed data sets, nonparametric tests were applied: Kruskal–Wallis for multiple comparisons and Mann-Whitney’s test with significance correction for double comparisons of independent samples. All measurements were expressed as mean  $\pm$  SEM. Values of  $p < 0.05$  were considered as significant.

## V. RESULTS

## **A. The cognitive signature of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice (9-month old)**

### **1. INTRODUCTION**

The determination of the mutations responsible for the familial forms of AD was an important step toward a better understanding of the molecular pathogenesis of AD. These mutations are found in genes that either code for the APP itself, which leads to an elevated production of soluble A $\beta$ <sub>1-40</sub> and insoluble A $\beta$ <sub>1-42</sub>, or for the enzymes PS1 or PS2, which results in an increased A $\beta$ <sub>1-42</sub>/A $\beta$ <sub>1-40</sub> ratio. The overexpression of genes related to familial forms of AD progressively induces A $\beta$  accumulation over several months in transgenic mice (Turner et al., 2003). Transgenic mice models that incorporate single APP mutations are characterized by a relatively slow accumulation of A $\beta$ , whereas double transgenic mice, with mutated genes for APP and PS1, accumulate A $\beta$  faster, develop plaques earlier, and show behavioural deficits earlier in life (Rowan et al., 2003; Sankaranarayanan, 2006; Eriksen and Janus, 2007).

In order to study the alteration of NMDAR function in AD (Part B), we used one of the most extensively used transgenic mice model of AD, the APP<sup>swe</sup>/PS1<sup>dE9</sup>. First described by Jankowsky et al. (2001), the APP<sup>swe</sup>/PS1<sup>dE9</sup> mice overexpress the Swedish mutation of APP, together with PS1 deleted in exon 9 (Jankowsky et al., 2001). It is already known that the APP<sup>swe</sup>/PS1<sup>dE9</sup> mice begin to develop A $\beta$  plaques by 4 months of age, starting in the cortex and invading the hippocampus by 9 months (Jankowsky et al., 2004). Plaques continue to increase up to 12 months of age (Garcia-Alloza et al., 2006a). Activated microglia and astrocytes are developed in parallel with A $\beta$  plaques deposition, especially in the vicinity of the A $\beta$  plaques. As for the other double transgenic model mice, we were not able to find any publication showing tangles-related-taupathology. In terms of amyloid deposition, males develop plaques more rapidly than females, reaching saturation at 9 months of age, whereas deposition in females does not reach a plateau until 12 months of age (Ordonez-Gutierrez et al., 2015).

The behavior of these animals has been partially characterized and summarize in the annex 2 of this thesis. Taken together, these 12-month old APP<sup>swe</sup>/PS1<sup>dE9</sup> mice seem to develop deficits in a widely used behavioral test measuring spatial navigation and reference

memory, the Morris water maze, specifically during acquisition of the hidden platform position and the related probe trial during which the hidden platform is removed from the pool, but not in the visible-platform test (Lalonde et al., 2005). They have been shown to commit more errors than wild-type mice in the Morris water maze at 13 months, but not at 7 months (Volianskis et al., 2010).

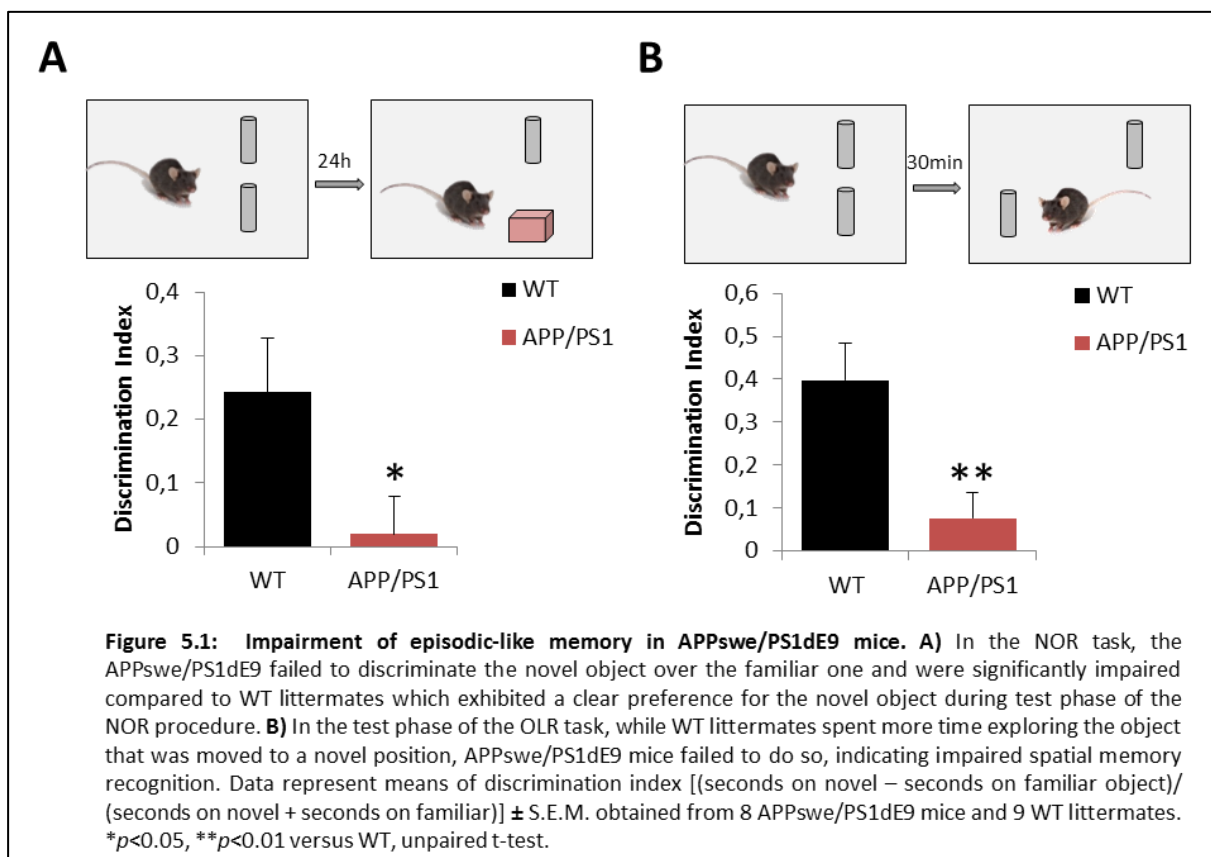
In order to further characterize the cognitive signature of APPswe/PS1dE9 mice and pinpoint specific cognitive deficits, we have used an innovative test battery composed of seven behavioural paradigms tailored to targeting hippocampal-dependent working, reference and episodic-like memory and specific memory processes (acquisition, consolidation, retrieval and flexibility). Because environmental enrichment has been used to manipulate the cognitive deficit in AD mice, we have also examined the impact of early or late environmental enrichment on cognitive signature of our APPswe/PS1dE9 mice. All these experiments have been conducted in 9-month old mice in which the A $\beta$  pathology is well-established (Jankowsky et al., 2004; Lagadec et al., 2012).

## 2. RESULTS

In order to characterize the cognitive signature of APPswe/PS1dE9 mice, we developed a battery of complementary tasks (Y-maze, 8-arm radial maze, novel object and novel location recognition) to pinpoint specific cognitive deficit (working, reference or episodic-like memory) in learning processes. To reduce the interaction between these different tasks, we have used two independent batches of mice, one for the innate tasks such as the Y-maze and the novel object/object location recognition and the second batch of mice for testing in the 8-arm radial maze.

Because the episodic memory function represents a core diagnostic hallmark of AD (Dubois et al., 2007) and precedes other cognitive deficits (Collie and Maruff, 2000; Bondi et al., 2008), we have first evaluated the performance of APPswe/PS1dE9 mice in the novel object recognition task, which is widely used for evaluating episodic-like memory in AD mouse models (Balducci et al., 2010; Lecoutey et al., 2014). This task exploits the innate tendency of rodents' spontaneous exploratory behaviour following a single-trial exposure to an event and, therefore, examines incidental (rather than motivated) learning, making it a more realistic model of human episodic memory processes than tasks which rely on

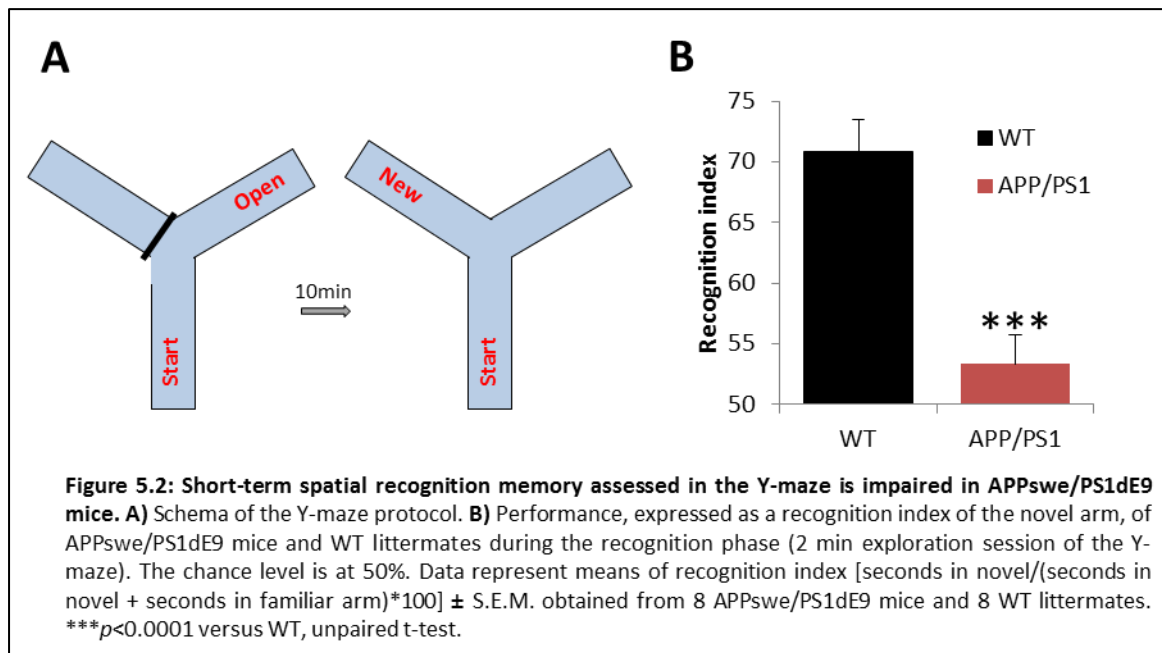
rewarded behavior (Dellu et al., 2000). Our object recognition test evaluated the “what” and “where” components of episodic-like memory. The “what” component is reflected by a greater exploration time for novel objects than familiar object, and is measured as the difference between the average exploration times of the familiar object and the novel object. We adapted the classical protocol of novel object recognition. To avoid confounding effect related to differential exploration time of objects during the acquisition phase, potentially leading to variations in initial memory strength across groups, we used the Ethovision™ video tracking system which was able to automatically stop the training when the exploration time of objects reached 1 min. Twenty-four hours later, during the retrieval phase, WT mice increased significantly the exploration time of the novel object while the APPswe/PS1dE9 mice spent a similar amount of time exploring both familiar and novel objects. The discrimination index calculated for both WT and APPswe/PS1dE9 groups reflected this differential pattern of exploration (Figure 5.1A).



Thus, WT mice had a higher discrimination index ( $p < 0.05$ ) compared to the APPswe/PS1dE9 mice (unpaired t-test,  $t_{(15)}=2.235$ ,  $p=0.041$ ).

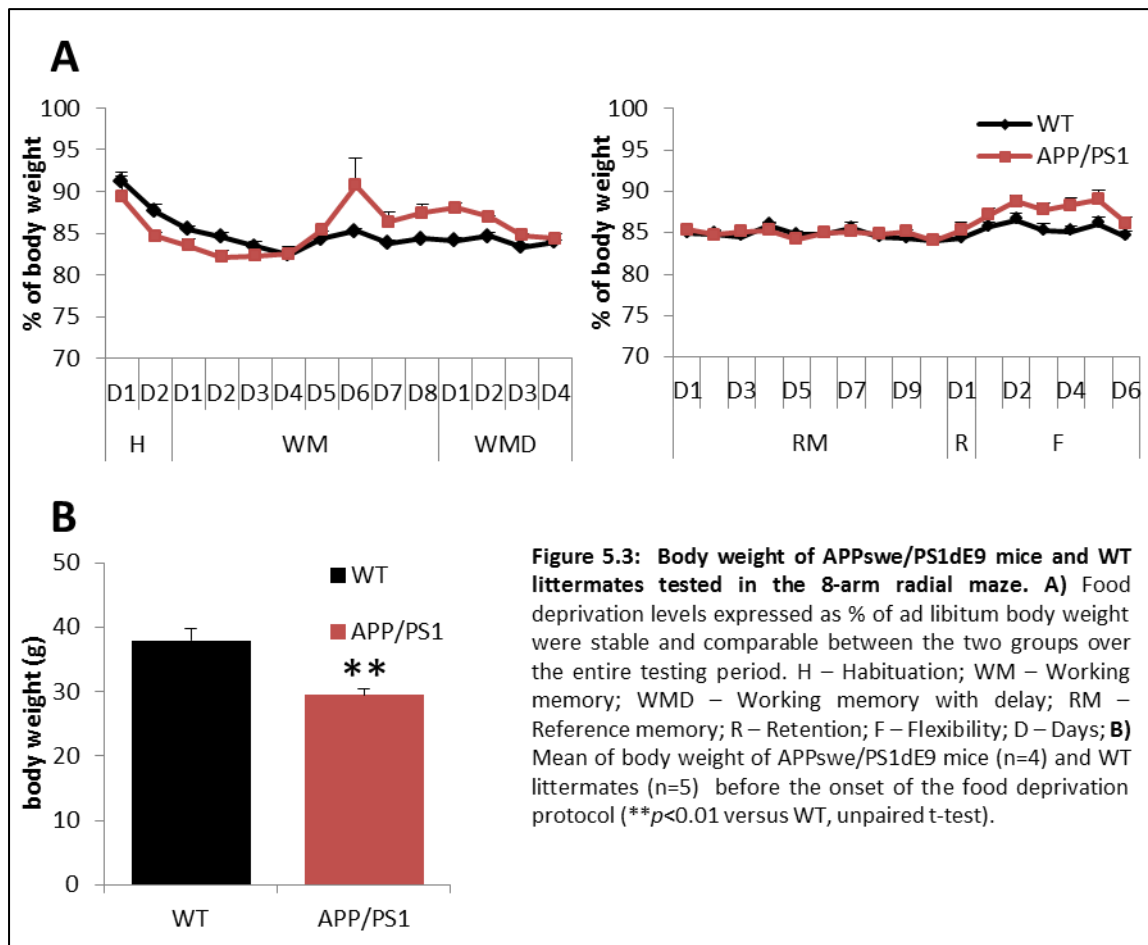
Similarly, we investigated the “where” component of episodic-like memory which refers to the memory for the specific places (locations) of objects. To this end, we used a spontaneous place recognition paradigm which taxes the spatial component of episodic-like memory (Ennaceur and Aggleton, 1997). This test is based on the innate tendency of mice to explore the object in a novel place compared with the object in a familiar place. Similarly to the novel object recognition paradigm, the exploration of objects during the acquisition phase was stopped when the amount of exploration time of both objects reached 1 min. Thirty minutes after the exploration phase (acquisition), we moved one object and assessed the exploration time of both objects. During the retrieval phase, WT mice increased significantly the exploration time of the object in the new position while the APP<sup>swe</sup>/PS1<sup>dE9</sup> mice spent a similar amount of time exploring both displaced and non-displaced objects, which resulted in a significantly different discrimination index between the WT and APP<sup>swe</sup>/PS1<sup>dE9</sup> groups (Figure 5.1B). Thus, WT mice exhibited a higher discrimination index ( $p < 0.05$ ) compared to APP<sup>swe</sup>/PS1<sup>dE9</sup> mice (unpaired t-test,  $t_{(15)} = 3.205$ ,  $p = 0.0059$ ).

Finally, to further assess spatial recognition memory in WT and APP<sup>swe</sup>/PS1<sup>dE9</sup> mice, we used the two-trial arm discrimination task in the Y-maze. As expected, after a single encoding phase of 5 min with only two arms accessible, a short ITI of 10 min resulted in a robust preference for the unexplored (previously closed) arm during the test phase (Figure 5.2) in WT mice. In contrast APP<sup>swe</sup>/PS1<sup>dE9</sup> mice failed to discriminate the novel arm (WT:  $70.88 \pm 2.61$ ,  $n = 9$ ; APP<sup>swe</sup>/PS1<sup>dE9</sup>:  $53.31 \pm 2.41$ ,  $n = 8$ , unpaired t-test,  $t_{(14)} = 4.949$ ;  $p = 0.0002$ ). This mutation-dependent impairment was memory-specific as there was no confounding effect of the mutation on the total exploration time of the open arms during the encoding phase.

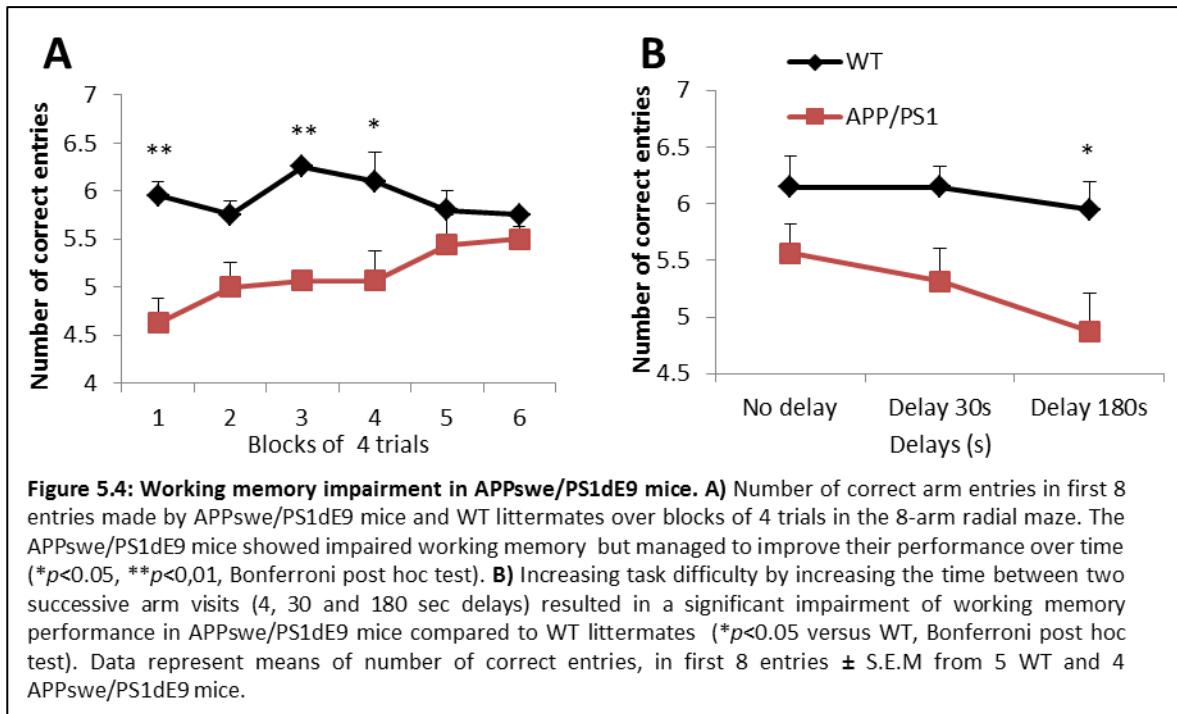


The second batch of WT and APPswe/PS1dE9 mice (9 months of age) was dedicated to examining spatial reference and working memory using the 8-arm radial maze, a classic test for investigating hippocampal-dependent working and reference memory in rodents (Crusio et al., 1993). Learning and memory performance in this maze requires intact spatial memory, and is impaired following hippocampal damage and aging (Britton et al., 2011). Because the learning process was motivated by food reward, the body weight of mice was assessed every day and stabilized at 85% of the *ad libitum* body-weight measured before the onset of the food deprivation protocol. As illustrated in Figure 5.3A and 5.3B, the percentage of body weight deprivation of APPswe/PS1dE9 mice during the course of working and reference memory testing in the 8-arm radial maze was not different from that of WT, suggesting that the level of motivation of APPswe/PS1dE9 mice could not explain the cognitive deficit observed in these mice.





Since tests of working memory can predict the time course and severity of AD in humans (Morris and Baddeley, 1988; Albert, 2011; Weintraub et al., 2012), we have first evaluated working memory in APPswe/PS1dE9 mice. Once handling and habituation to the 8-arm radial maze was completed, mice were trained daily using a working memory paradigm (Figure 5.4). In this paradigm, all 8 arms of the maze were baited with condensed milk pellets. Mice were required to retrieve food from all arms of the maze and the arm re-entry (repeated visits to an already food-depleted arm) was automatically counted as an error. Care was taken to ensure that animals did not solve the maze in ways other than using working memory, such as for instance via the use of a serial “sweeping” strategy (entering each arm successively in a clockwise or counter-clockwise manner). Thus, to prevent the use of a non-mnemonic strategy, all doors were closed for 4 seconds between each arm exploration.

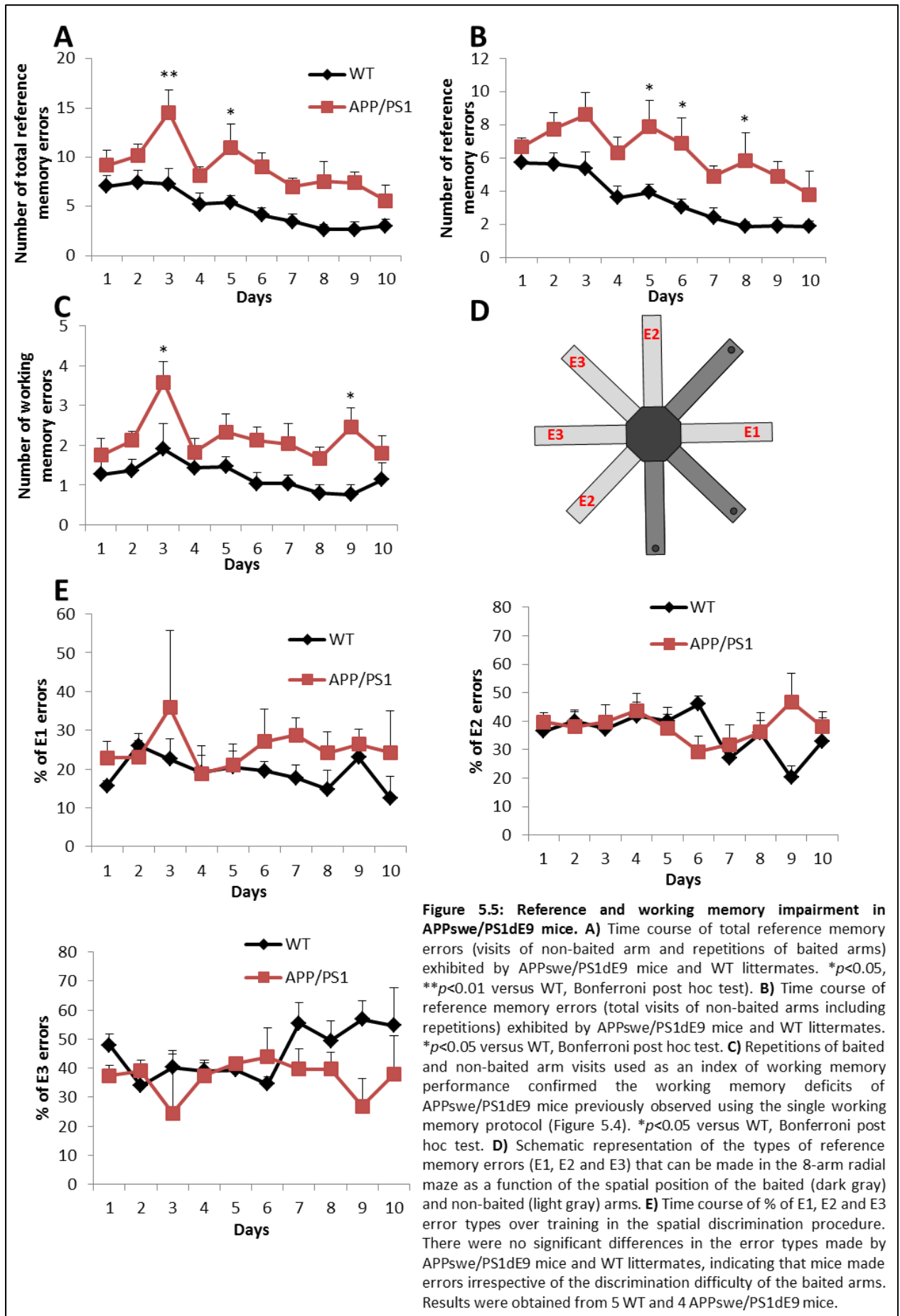


We compared the cognitive performance of WT and APPswe/PS1dE9 mice by analysing the number of correct choices for the first 8 entries for each trial. Working memory assessment occurred over 4 days in which each mouse was tested every day (2-4 trials/day) with a 1-h rest period between trials (the mouse returned to its home cage). The results illustrated in Figure 5.4A show an overall significant deleterious effect of the transgene (two-way ANOVA; transgene effect:  $F_{(1,42)} = 32.07$ ,  $p < 0.0001$ ) on working memory performance. A significant post-hoc effect at the beginning of training (for block of trials 1 and 3, Bonferroni post-hoc test  $p < 0.01$  and for block of trial 4, Bonferroni post-hoc test  $p < 0.05$ ) was observed, but not later on, indicating that the working memory deficit initially observed in APPswe/PS1dE9 mice could be progressively reversed by increasing the amount of training (Figure 5.4A). To evaluate the impact of temporal interference on working memory performance, we added a delay (30 sec or 180 sec) between two arm choices after completion of 4 correct choices. When submitted to these delays, WT and APPswe/PS1dE9 mice exhibited similar performances (4 days training). Four sec delay sessions were used as controls to assess delay effects. Increasing the delay between arm visits from 4 to 180 seconds significantly reduced the performance of APPswe/PS1dE9 mice whereas WT mice were unaffected and continued to exhibit similar performance even for the 180 sec delay condition (Figure 5.4B, Bonferroni post-hoc test;  $p < 0.05$ ). Two-way ANOVA revealed a significant effect of the genotype (two-way ANOVA; transgene effect:  $F_{(1,21)} = 14.67$ ,  $p = 0.001$ ). Altogether, these findings

demonstrate that the working memory performance of APPswe/PS1dE9 mice is impaired, exacerbated when a delay is added (delay-dependent) and reversible by training.

After completion of the working memory session, all mice were re-trained on the 8-arm radial maze using a new configuration of 3 baited and 5 non-baited arms (reference memory paradigm). The results presented in Figure 5.5A show an overall significant effect of transgene on the total number of errors, defined as the number of entries in the non-baited arms and the repetition of entries into baited arms (two-way ANOVA; transgene effect:  $F_{(1,70)} = 53.17, p < 0.0001$ ; time effect:  $F_{(9,70)} = 5.44, p < 0.0001$ ).

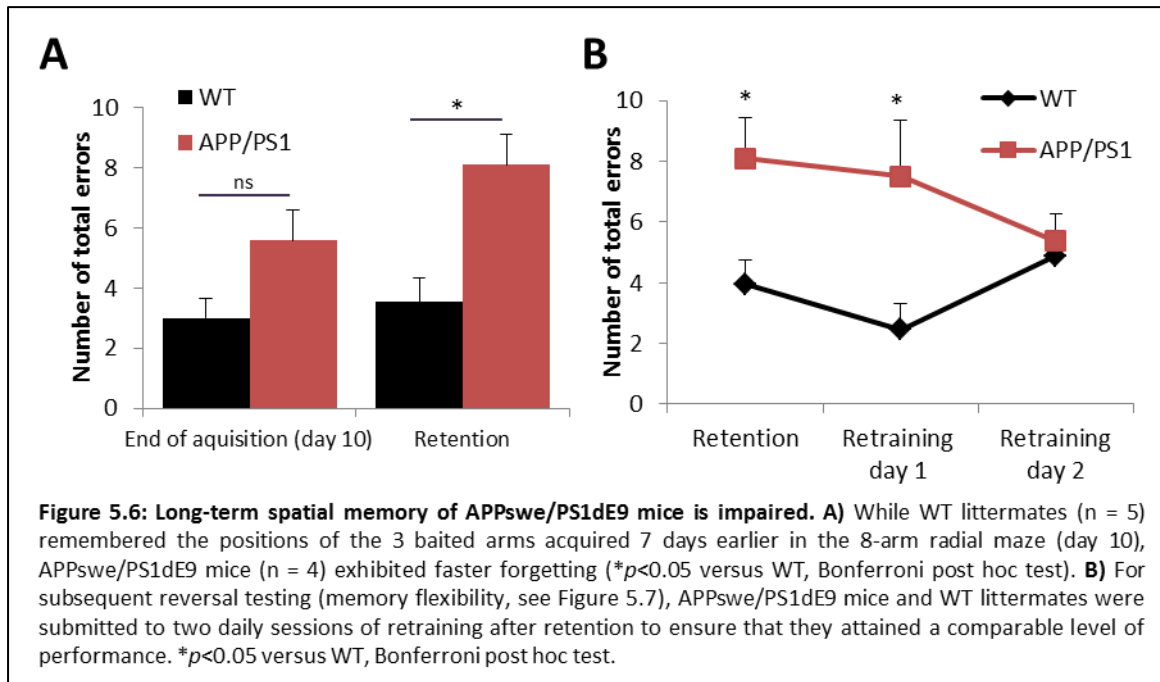
Whereas the animals were required to memorize which set of three arms were baited across trials (spatial reference memory), the animals also needed to memorize the arms that had already been visited during a given trial from those that had not (spatial working memory) to achieve the optimal performance in this task. Because the total errors in this task are dependent on both types of memory (reference and working), we next analysed reference memory and working memory errors separately. Two-way ANOVA of reference memory errors showed a significant reduction in reference memory errors over training days ( $F_{(9,70)} = 5.39, p < 0.0001$ ) but also a significant effect of transgene ( $F_{(1,70)} = 49.67, p < 0.0001$ ), indicating that while both WT and APPswe/PS1dE9 were able to learn the task (transgene x days interaction,  $F_{(9,70)} = 0.6725; p = 0.7309$ ), APPswe/PS1dE9 mice committed more errors and were slower in finding the three baited-arms and achieving task mastery. A similar observation stands for working memory (two-way ANOVA; transgene effect:  $F_{(1,70)} = 29.07, p < 0.0001$ ; time effect:  $F_{(9,70)} = 2.13, p = 0.0379$ ; confirming the previously observed results when all arms were baited.



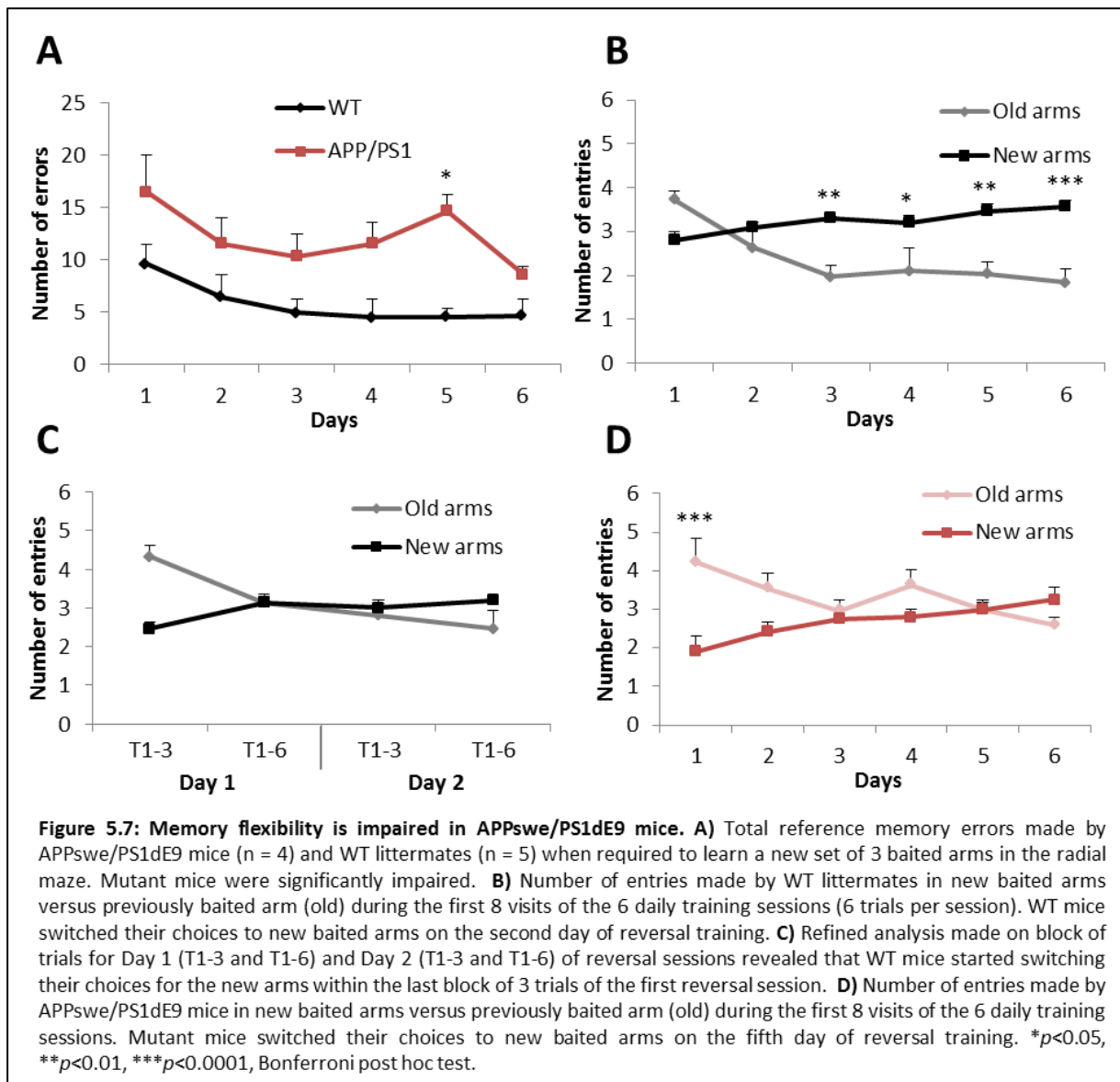
**Figure 5.5: Reference and working memory impairment in APPswe/PS1dE9 mice.** **A)** Time course of total reference memory errors (visits of non-baited arm and repetitions of baited arms) exhibited by APPswe/PS1dE9 mice and WT littermates. \* $p < 0.05$ , \*\* $p < 0.01$  versus WT, Bonferroni post hoc test). **B)** Time course of reference memory errors (total visits of non-baited arms including repetitions) exhibited by APPswe/PS1dE9 mice and WT littermates. \* $p < 0.05$  versus WT, Bonferroni post hoc test). **C)** Repetitions of baited and non-baited arm visits used as an index of working memory performance confirmed the working memory deficits of APPswe/PS1dE9 mice previously observed using the single working memory protocol (Figure 5.4). \* $p < 0.05$  versus WT, Bonferroni post hoc test). **D)** Schematic representation of the types of reference memory errors (E1, E2 and E3) that can be made in the 8-arm radial maze as a function of the spatial position of the baited (dark gray) and non-baited (light gray) arms. **E)** Time course of % of E1, E2 and E3 error types over training in the spatial discrimination procedure. There were no significant differences in the error types made by APPswe/PS1dE9 mice and WT littermates, indicating that mice made errors irrespective of the discrimination difficulty of the baited arms. Results were obtained from 5 WT and 4 APPswe/PS1dE9 mice.

In our reference memory paradigm, mice had to discriminate non-baited arms that were surrounded by two baited arms, adjacent to baited-arms or surrounded by two non-baited arms. Thus, positions of these non-baited arms make them more or less difficult to discriminate. Accordingly, we defined different error types (E1-most difficult; E2-intermediate; E3-easier) based on the respective positions of these baited arms (see Figure 5.5D). We analysed the number of errors as a function of training days and according to their types (E1, E2 and E3). As illustrated in Figure 5.5E, only the number E3 type errors was significantly different between WT and APPswe/PS1dE9 mice (two-way ANOVA; transgene effect:  $F_{(1,70)} = 4.153$ ,  $p < 0.05$ ). The error types E1 and E2 were not significantly different between WT and APPswe/PS1dE9 mice (two-way ANOVA; transgene effect:  $F_{(1,70)} = \text{NS}$ ,  $p > 0.05$ ), suggesting that the overall higher number of errors made by APPswe/PS1dE9 mice during training was not related to the intrinsic discrimination difficulty of the baited arms.

We next examined whether one component of this apparent “learning deficit” included a potential increase in the rate of forgetting. This requires WT and APPswe/PS1dE9 mice to have an equivalent level of performance upon completion of the acquisition phase. This was indeed the case since both WT and APPswe/PS1dE9 mice exhibited signs of learning and achieved a rather similar performance after 10 days of training (Bonferroni post-hoc test;  $p > 0.05$ ). Thus, reaching this criterion set the stage for the next phase of testing. A retention test was then administered—without intervening training—to examine forgetting. As illustrated in Figure 5.6A, 7 days after the last day of training, the APPswe/PS1dE9 mice made more errors to find the set of 3 baited arms compared to WT, indicating that these mice forgot the spatial positions of the baited arms. Whereas the number of errors performed by WT mice to find the baited-arms were similar before and after the delay (two-way ANOVA; transgene effect:  $F_{(1,14)} = 11.02$ ,  $p = 0.0051$ ).



Finally, we also examined if APPswe/PS1dE9 mice had the ability to adapt when the positions of the baited arms in the radial arm maze were modified (reversal protocol in which spatial contingencies of baited arms changed). After the retention test, we thus re-trained the mice for another 2 days. At this point of training, both groups reached a similar level of performance (Figure 5.6B; two-way ANOVA; transgene effect:  $F_{(1,21)} = 13.71$ ,  $p < 0.05$ ). The total number of errors made by APPswe/PS1dE9 mice during reversal sessions was higher compared to WT mice (Figure 5.7A; two-way ANOVA; transgene effect:  $F_{(1,42)} = 33.32$ ,  $p < 0.0001$ ; training days effect:  $F_{(5,42)} = 2.73$ ,  $p = 0.0316$ ). In order to determine the ability of mice to learn the new spatial contingency, we also compared the number of entries in newly baited arms versus previously baited-arms during the first 8 entries of each of the 6 daily reversal sessions (Figure 5.7B, C, D). During the reversal phase, WT mice learned the task significantly faster than APPswe/PS1dE9. Indeed WT mice already switched to the new set of baited arms after the first day of training (Figure 5.7C). In contrast, APPswe/PS1dE9 mice which needed at least five days to acquire the new spatial contingency (Figure 5.7D), suggesting a deficit in their ability to adapt flexibly to a novel situation.

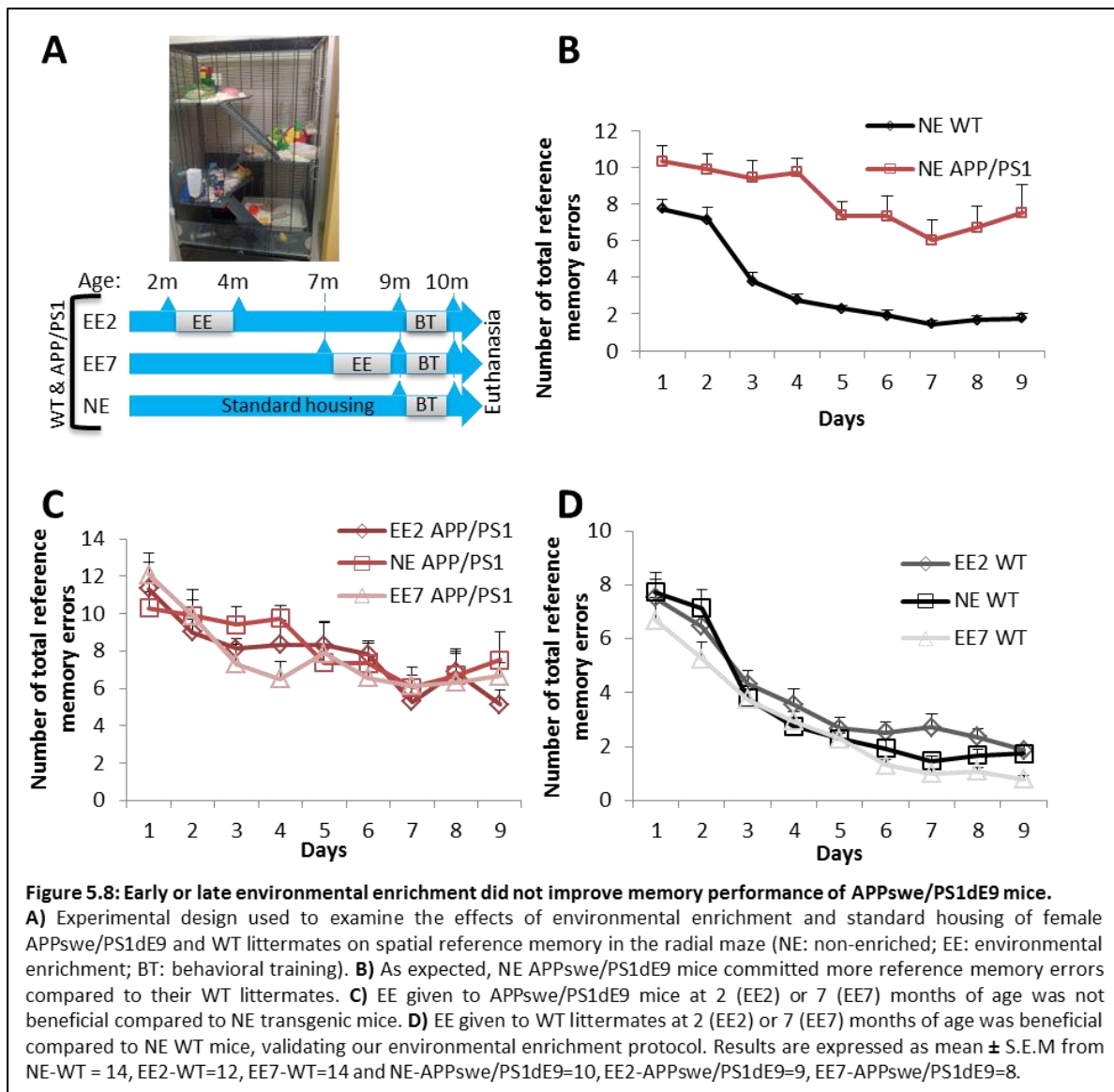


Altogether, by using the 8-arm radial maze, we are able to show that 9-month old APPswe/PS1dE9 mice exhibit a severe cognitive deficit that affects both working and reference memory. These mice exhibit slower learning, faster forgetting and lower flexibility when confronted to a change of spatial contingencies.

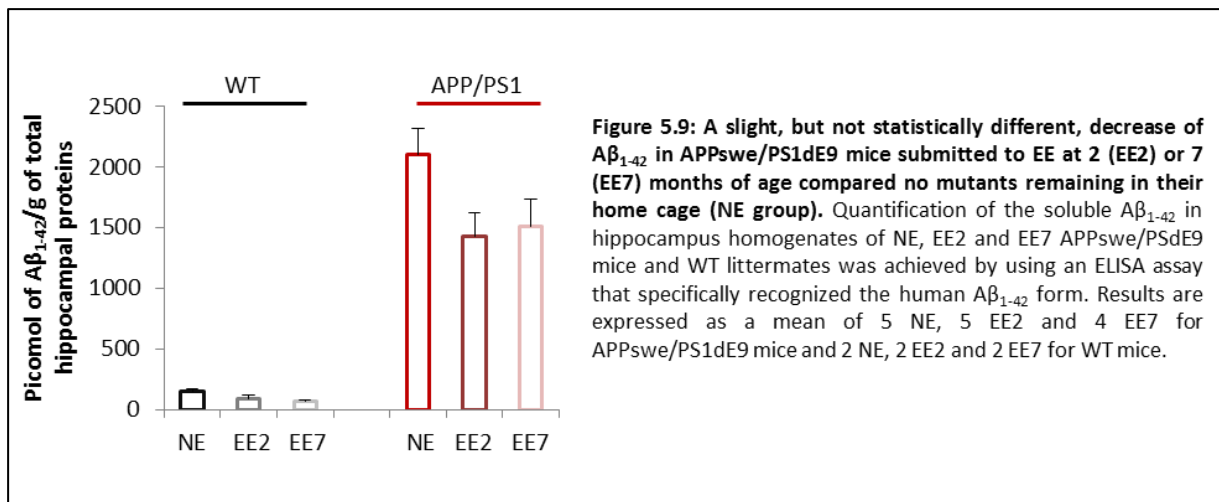
A large body of evidence indicates that environmental enrichment (EE) not only improves learning and memory performance in healthy animals (van Praag et al., 2000; Bruel-Jungerman et al., 2006), but also is able to mitigate cognitive decline in transgenic models of familial AD (Arendash et al., 2004; Jankowsky et al., 2005; Wolf et al., 2006; Costa et al., 2007; Cracchiolo et al., 2007; Hu et al., 2010; Valero et al., 2011). To evaluate the sensitivity of our APPswe/PS1dE9 mouse model to the EE and because the exposure to EE

during early adulthood (before amyloidogenesis occurs) reduces the severity of AD-related cognitive deficits more efficiently than exposure later in life (when the pathology is already present), we compared the effect of early (2-month old mice) and late (7 months old mice) enrichment on cognitive deficit in our 9-month old transgenic mice. EE consisted of a large box (100×70×40cm), containing various toys, wooden blocks, climbing platforms, plastic tubes, small houses, but no running wheel. The toys, food and water were rearranged and renewed every three days to stimulate animals' exploratory behavior. Mice were exposed to the enriched environment by groups of 10 to 15. Animals assigned to the non-enriched (NE) group remained in standard laboratory cages by groups of 5 for the entire duration of the experiment. In both housing conditions, animals had free access to food and water. After the 10-week period of enrichment, mice were returned to their home cage where they remained until the age of 9 months. Their behavior was then tested. Testing started with the 8-arm radial maze to evaluate reference memory (Figure 5.8). As previously observed, results illustrated in Figure 5.8B for the total number of reference memory errors between non enriched WT and APP<sup>swe</sup>/PS1<sup>dE9</sup> mice showed an overall significant effect of transgene (two-way ANOVA; transgene effect:  $F_{(1,198)}=218.9$ ,  $p<0.0001$ ; training days effect:  $F_{(8,198)}=14.94$ ,  $p<0.0001$ ). As expected, two-way ANOVA of total errors in WT mice exposed to EE showed that days of training ( $F_{(8,288)}=65.96$ ,  $p<0.0001$ ) and EE ( $F_{(2,288)}=8.678$ ,  $p=0.0002$ ) were able to reduce the total errors during training, validating our environmental enrichment as pertinent to improve memory (Figure 5.8D). As previously observed, two-way ANOVA of total errors in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice (Figure 5.8C) showed an effect of training days ( $F_{(8,192)}=8,262$ ,  $p<0.0001$ ), but surprisingly no effect of early (EE2) or late (EE7) EE ( $F_{(2,192)}=0.376$ ,  $p=0.6962$ ).





Because previous studies have demonstrated that EE was able to slightly reduce brain amyloid charge, we have also quantified the amount of soluble  $A\beta_{1-42}$  in hippocampus of NE, EE2 and EE7 APPswe/PSdE9 mice by using an ELISA assay that specifically recognized the human  $A\beta_{1-42}$  form. As illustrated in Figure 5.9, we observed a slight but not statistically significant decrease in the amount of  $A\beta_{1-42}$  in both EE2 and EE7 groups compared to NE group (because of the small sample size, we used the non-parametric Kruskal-Wallis test,  $p=0.1205$ , NE group  $n=5$ ; EE2 group  $n=4$ ; EE7 group  $n=4$ ). This data suggest that the APPswe/PSdE9 mouse model at this age is not suitable to study the impact of a cognitive reserve and/or environmental enrichment. The improvement of cognitive performance could be tested before 9 months at an earlier stage of the pathology (4-6 months).



### 3. DISCUSSION

The APPswe/PS1dE9 model was designed to mimic, in mice, the symptoms of AD typically observed in humans. As with any animal model of a human disease, it is unlikely that all behaviors will be exactly replicated, however the more closely a model resembles the disease pathology; the more likely researchers will be able to develop pertinent treatments for humans afflicted by the disease (Webster et al., 2014). To further explore the potential of this rodent analogue model of AD, we have developed a battery of behavioral tasks (novel object recognition task, novel place object recognition, Y-maze, 8-arm radial maze) used to investigate various cognitive domains with relevance to AD and established the cognitive signature of the APPswe/PS1dE9. In the present set of experiments, we have demonstrated that APPswe/PS1dE9 mice (9-month old) develop spatial and non-spatial memory deficits. Indeed, APPswe/PS1dE9 mice exhibit a severe episodic-like memory deficit since these mice were not able to identify a new object or a new place of the familiar one. For the first time, we have also used the 8-arm radial maze to evaluate working and reference memory in APPswe/PS1dE9 mice. By using a sequence of tasks, we were able to show that APPswe/PS1dE9 mice are slow learners when submitted to both working and reference memory paradigms. When reaching the same level of performance, APPswe/PS1dE9 mice also forget more quickly than age-matched WT mice. We have also found that APPswe/PS1dE9 mice have a low cognitive flexibility. Increasing the cognitive reserve of these mice early (at 2 months of age) or later (at 5 months of age) did not reverse their cognitive deficits and did not affect soluble Aβ<sub>1-42</sub> levels.

Among the early cognitive symptoms of AD, short-term episodic memory impairment associated with attention and spatial orientation disturbances have been mainly described (Snowden et al., 2011). Impaired visual recognition memory reflecting dysfunction of the anterior subhippocampal cortex (transentorhinal, entorhinal and perirhinal cortices), has been proposed as an early marker for AD diagnosis (Didic et al., 2010). Assessed through the object or place recognition paradigms in animals, visual recognition memory is mainly supported by cortical interactions involving the perirhinal and entorhinal cortices, while the hippocampus is predominantly involved in processing the spatial and temporal context of recognition (Mumby and Pinel, 1994). Recognition memory has been particularly relevant in the field of AD research which may explain why deficits in recognition have been studied and observed in almost all transgenic mouse models of AD. Indeed, the novel object recognition test has been used for the detection of memory deficit in single, double or triple transgenic mice expressing mutation in APP, PS1 or/and tau (Grayson et al., 2015). In our experiments, we have shown that memory of a single episode (object recognition, spatial location of object or spatial memory) is heavily impaired in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice which is in line with previous findings in 5/6 month-old APP<sup>swe</sup>/PS1<sup>dE9</sup> mice (Sierksma et al., 2013; Sierksma et al., 2014; Petrov et al., 2015) but also in older (13 months) mice or late (Koivisto et al., 2014; Li et al., 2014). The variability in the cognitive profile observed in these mice could be explained by the diversity of the protocols employed (e.g. varying retention intervals between encoding and testing) but also by the genetic background of the animals studied.

If individuals affected by AD first exhibit subtle deficits in episodic memory, the impairment of working memory and eventually long-term memory deficits can predict the shift from mild cognitive impairment to AD and the rate at which AD progresses with age (Morris and Baddeley, 1988; Albert, 2011; Weintraub et al., 2012). In rodents, working memory is a transient form of memory that serves to carry out a specific task in a given time, but not typically between training sessions occurring over 24 hour periods. It is distinguishable from reference memory, which is a memory that is typically acquired with repeated training, and persists from days to months, and is therefore useful to evaluate long-term deficit (Bontempi et al., 1999). To evaluate working and reference memory in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice, we have used the 8-arm radial maze. Indeed this apparatus has been used for many years by numerous laboratories, and there is a consensus in the field of

behavioral neuroscience that it measures working memory in mice with a strong spatial component. Paradoxically, it is not as widely used in the Alzheimer's field as the Morris water maze (12 citations in PubMed for keyword "8-arm radial maze"–"Alzheimer" compared to 797 for keyword "water maze"–"Alzheimer"). Compared to the water maze, the 8-arm radial offers however a number of advantages: 1/ mice can perform poorly in the water maze (Gerlai and Clayton, 1999), partially due to the fact that their performance can be affected by thigmotaxis (the tendency to swim near the wall of the maze) and passivity (mice tend to float more compared to rats) (Upchurch and Wehner, 1988; Vorhees and Williams, 2006); 2/ levels of stress are likely to be higher in the aversive water maze than in the 8-arm radial maze which is appetitive in nature. Indeed failure to find a food reward incurs no great penalty, whereas failure to find the hidden platform is potentially life threatening; 3/ the 8-arm radial maze is less sensitive to a search-strategy than the water maze since mice are required to make a choice between arms. It is therefore more useful for apprehending potential changes of error patterns than searching patterns; thus, in our study, we have observed that the acquisition for working and reference memory are slower and delay-dependent. Although no one used the radial maze as we did, several studies in APPswe/PS1dE9 mice have been performed using a radial arm water maze (RAWM), in which the goal of the mouse is to escape from water by finding a submerged platform hidden at the end of one of the arms. Results are controversial, especially with respect to the age at which the radial maze task detects a working memory deficit. In APPswe/PS1dE9 mice, some studies demonstrated impairment in RAWM performance starting at 6 months of age (Xiong et al., 2011), whereas, in other articles, performances of transgenic mice were normal at 7 months of age and reduced only at 13 months (Volianskis et al., 2010) or at 10–15 months (Sood et al., 2007).

We have also found a reference memory deficit in APPswe/PS1dE9 mice which is in line with previous findings that have relied on the Morris water maze. Several groups have shown a cognitive impairment of APPswe/PS1dE9 mice between 6 and 9-month of age (Toth et al., 2013; Wang et al., 2013a; Jiang et al., 2014; Li et al., 2014; Ramos-Rodriguez et al., 2014; Tan et al., 2014; Wang et al., 2014a; Xu et al., 2014) or even earlier (Kemppainen et al., 2014; Gao et al., 2015). Other groups have shown a clear cognitive deficit in 12 month-old

mice or even later (Savonenko et al., 2005; Hooijmans et al., 2009; Zhang et al., 2011b; Vollert et al., 2013; Koivisto et al., 2014; Janus et al., 2015).

Most of the studies in the AD field have focused on understanding the intricacies of the encoding deficit in patients with AD or in animal models of AD, from the molecular to the cognitive level, in an attempt to exploit areas of intact functioning and perhaps provide targets for early behavioral or pharmacological intervention. However, some human studies have provided supportive evidence that AD could be a disorder of rapid forgetting (Hart et al., 1988; Salmon et al., 1989; Carlesimo et al., 1993; Reed et al., 1998; Ally et al., 2013). This abnormal forgetfulness can be reliably pinpointed in early phases of AD using tests of delayed recall (Welsh et al., 1991) and can be detectable prior to the onset of AD. There is a large degree of overlap between the concepts of encoding disorder and rapid forgetting. One might assume that if information is not properly encoded, the resulting memory is rapidly or easily forgotten. In our study, we were able to analyze the rate of forgetting because APP<sup>swe</sup>/PS1<sup>dE9</sup> and age-matched WT mice managed to attain a similar level of initial learning. As previously observed in another mouse model of AD (Daumas et al., 2008), we provide converging evidence that APP<sup>swe</sup>/PS1<sup>dE9</sup> mice could be used to study accurately the rate and the molecular mechanisms of forgetting. Overall, we have been able to establish a cognitive signature of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice by using a battery of tasks which covers various cognitive domains with relevance to AD and by using a limited number of mazes to reduce the confounding effect of stress.

The second part of this project was to identify if an early or late environmental enrichment might slow-down the cognitive deficit in our APP<sup>swe</sup>/PS1<sup>dE9</sup> as previously observed by several groups in other AD mice models (Arendash et al., 2004; Jankowsky et al., 2005; Lazarov et al., 2005; Costa et al., 2007; Cracchiolo et al., 2007; Verret et al., 2013). Surprisingly, we were not able to detect difference between the cognitive performance of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice with or without early or late environmental enrichment in the 8-arm radial maze. Since we detected an effect of late environmental enrichment in age-matched WT mice, it is unlikely that the lack of effect in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice can be attributed to our enrichment protocol.

The lack of effect could be due to the model of AD mice used: single versus double transgenic mice. Indeed several studies showed that PS1 mutations might affect the cognitive and molecular (neurogenesis) benefits of environmental enrichment (Jankowsky et al., 2003; Veeraraghavalu et al., 2013; Veeraraghavalu and Sisodia, 2013) or even exacerbated amyloid plaque formation (Cotel et al., 2012). Another possible explanation could be that the development of AD pathology (plaques, inflammation, synaptic loss...) in double transgenic mice was too fast. Indeed 9-month old APP<sup>swe</sup>/PS1<sup>dE9</sup> mice have pathological hallmarks widely distributed and considered as a model of late phase of AD pathology. However the impact of environmental enrichment is mainly observed at beginning of pathological process and delays the appearance of AD symptoms (Berardi et al., 2007). Thus, we could not exclude that we missed the benefits of enrichment in our 9-month old mice. In this line, the quantification of soluble A $\beta$ <sub>1-42</sub> in hippocampal homogenates has revealed a slight (but not significant) reduction of soluble of A $\beta$ <sub>1-42</sub>, and surprisingly in both EE2 and EE7 groups, suggesting that environmental enrichment could slow-down A $\beta$  production and/or increase A $\beta$  clearance as suggested by Verret et al. (2013).

Taken together, we have obtained a clear cognitive signature of APP<sup>swe</sup>/PS1<sup>dE9</sup>, which could be used to correlate the specific cognitive domain and molecular and/or structural alterations in order to identify or characterize pathological processes involved in the AD.

## **B. NMDAR subcellular location and memory functions are altered in the APP<sup>swe</sup>/PS1<sup>dE9</sup> mouse model of Alzheimer's disease**

### **1. INTRODUCTION**

Synaptic loss is an early and invariant feature of AD and a strong correlation between the extent of synaptic loss and the severity of dementia in human patients has been reported (Roth *et al.*, 1966; Terry, 1996). Although our understanding of how and what forms of A $\beta$  mediate synaptic dysfunction and later on synaptic loss is incomplete, there is a growing consensus that it is the oligomeric form of A $\beta$  which predominantly interacts with the post-synaptic density either directly or indirectly with one or more synaptic receptors and initiates transduction mechanisms which in turn result in decreased synaptic plasticity and synaptotoxicity (Shankar and Walsh, 2009).

Since the initial findings that NMDARs play important roles in cellular models of learning as well as synaptotoxicity and neurotoxicity, abnormal function of these receptors has been considered as a potential mechanism in the pathophysiology underlying Alzheimer's disease (AD) (Malinow, 2012). There are several potential roles for the NMDARs in A $\beta$ -related mechanisms: first, the NMDAR may be a receptor for A $\beta$ , or it may associated indirectly, by interacting with molecules that bind A $\beta$  (Cisse *et al.*, 2011); second, NMDARs may be necessary, either by mediating or acting permissively, in the actions of A $\beta$  on synaptic transmission and plasticity (Hu *et al.*, 2009; Rammes *et al.*, 2011; Ronicke *et al.*, 2011); third, NMDAR function may be an important downstream target of A $\beta$ , that is, A $\beta$  may cause a reduced or enhanced function of NMDARs (Hsia *et al.*, 1999; Kamenetz *et al.*, 2003; Snyder *et al.*, 2005; Harris *et al.*, 2010).

Over the past decade, a recent "localization hypothesis of NMDARs" has suggested that overactivation of NMDARs located outside of the synapse, the so-called "extrasynaptic area", plays a major role in neuronal dysfunction and death, whereas physiological activation of these NMDARs inside the synapse, the so-called "synaptic area", can contribute to cell survival and plasticity (Hardingham and Bading, 2010). Recent evidence supports a significant role for enhanced extrasynaptic activity in the pathogenesis of AD. A $\beta$  negatively regulates the number of NMDARs at postsynaptic sites, thereby disrupting the balance

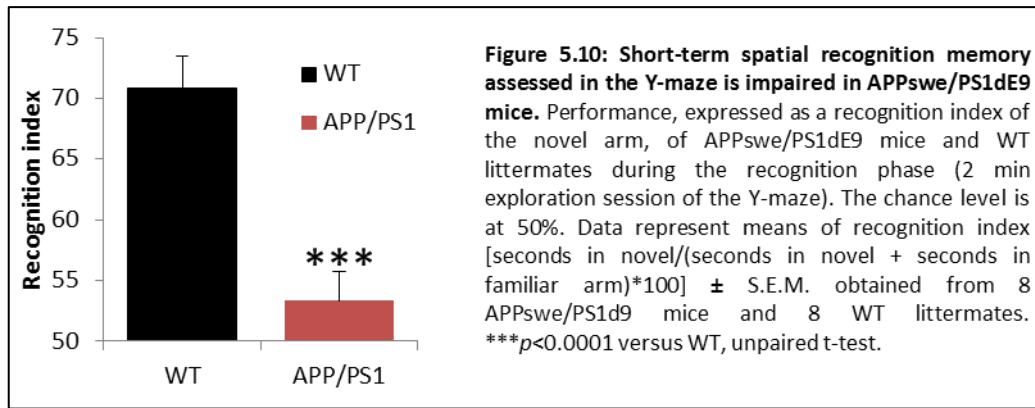
between synaptic and extrasynaptic NMDAR activity (Snyder et al., 2005; Li et al., 2011). Memantine, which has shown a clinical benefit in late phases of AD with an excellent clinical safety profile, effectively blocks excessive extrasynaptic NMDAR-mediated currents, while relatively sparing normal synaptic activity (Xia et al., 2010).

Thus, NMDAR subcellular location and/or over-activation of extrasynaptic NMDAR could impair the plasticity-related events triggered by learning and memory processes. To test this hypothesis, we have adopted an integrative strategy which favors vertical levels of analyses (from phenotype to molecular events) and combines a set of interrelated correlative and invasive approaches in a double transgenic mouse model of AD (APP<sup>swe</sup>/PS1<sup>dE9</sup>) and their age-matched wild-type (WT) controls. Altogether, our results identify the increase in the extrasynaptic pool of GluN2B-containing NMDARs as a deleterious mechanism which translates into impaired memory functions and suggest that A $\beta$ s can exert their deleterious effect by destabilizing the synaptic spatial distribution of NMDAR subunits.

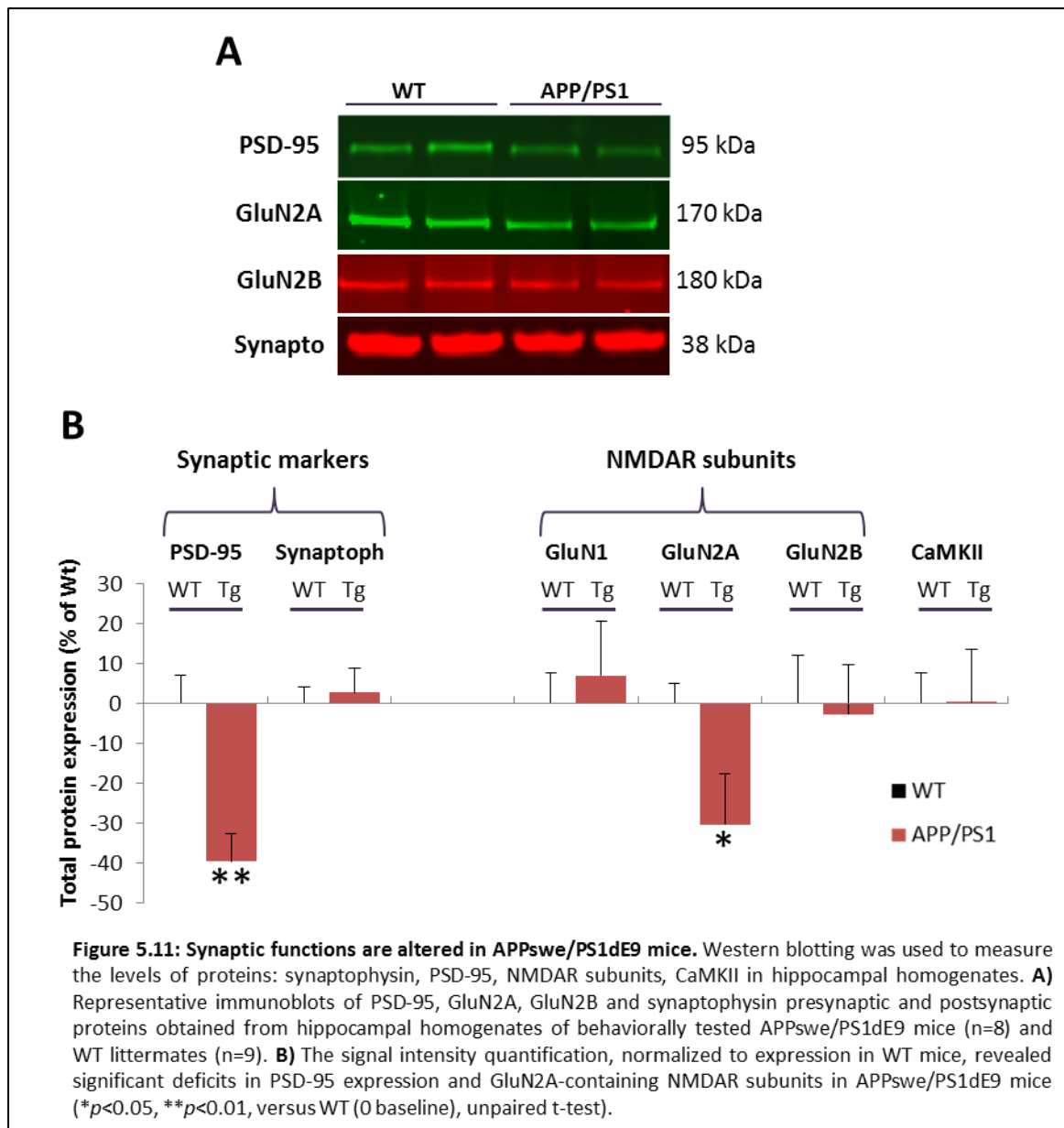
## 2. RESULTS

Before analyzing the patterns of protein expressions in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice, we first established the cognitive performance of 8 WT and 9 APP<sup>swe</sup>/PS1<sup>dE9</sup> mice by using the classical version of the Y-maze (spatial discrimination procedure). After a single encoding phase of 5 min with only two arms accessible, a short ITI of 10 min resulted in a robust preference for the unexplored (previously closed) arm during the test phase (Figure 5.10, similar to Figure 5.1) in WT mice. In contrast, APP<sup>swe</sup>/PS1<sup>dE9</sup> mice failed to discriminate the novel arm (WT:  $70.88 \pm 2.61$ ,  $n=9$ ; APP<sup>swe</sup>/PS1<sup>dE9</sup>:  $53.31 \pm 2.41$ ,  $n=8$ , unpaired t-test,  $t_{(14)}=4.949$ ;  $p=0.0002$ ). This mutation-dependent impairment was memory-specific as there was no confounding effect of the mutation on the total exploration of the open arm during the encoding phase. Twenty four hours later, mice were euthanized to determine the changes in synaptic protein resulting from the spatial discrimination challenge in the Y-maze. We focused our analysis in the hippocampus which is specifically recruited in this task and we separately analyzed each hippocampus for each animal, one for total protein expression and the other one for fractionation.





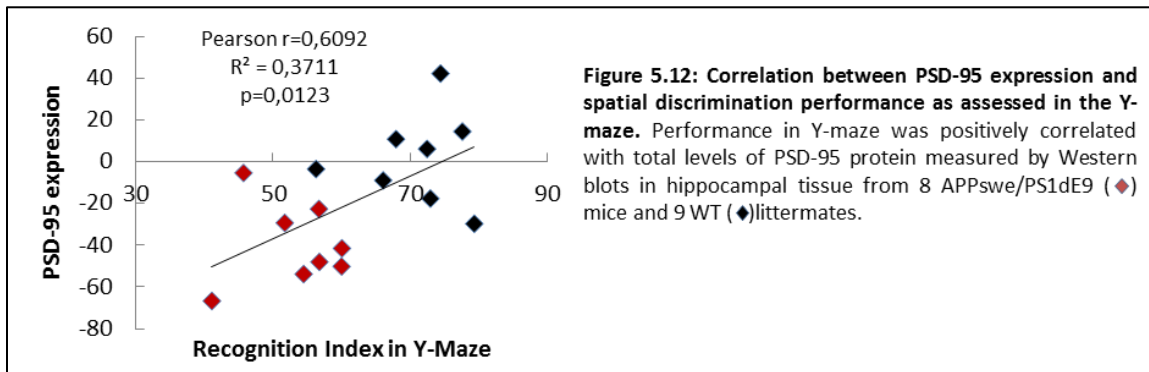
We studied the protein expression level of the presynaptic marker synaptophysin, the post-synaptic marker PSD-95 but also the GluN2A, GluN2B, GluN1 subunits of NMDARs and the calcium/calmodulin-dependent protein kinase II (CaMKII). The same amount of hippocampal proteins was separated by SDS-PAGE electrophoresis and the expression of synaptic markers determined by Western blots (Figure 5.11). A densitometric analysis of band intensity was performed. Results were calculated as percentage of protein expression relative to WT control mice (0% baseline). As illustrated in Figure 5.11A and 5.11B, we found a significant decrease of PSD-95 protein expression in the hippocampus of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice (APP<sup>swe</sup>/PS1<sup>dE9</sup>: -39.71% ± 6.92 compared to controls, *n*= 8-9, unpaired t-test,  $t_{(15)}=3.98$ ; *p*=0.0012) as well as GluN2A (APP<sup>swe</sup>/PS1<sup>dE9</sup>: -30.37% ± 12.77, *n*= 8-9, unpaired t-test,  $t_{(15)}=2.314$ ; *p*=0.035). Surprisingly, the GluN2B subunit of NMDARs was not altered (APP<sup>swe</sup>/PS1<sup>dE9</sup>: -2.6% ± 12.41 *n*= 8-9, unpaired t-test,  $t_{(15)}=0.1488$ ; *p*=0.8837). The expressions of GluN1 (APP<sup>swe</sup>/PS1<sup>dE9</sup>: 7.069%±13.74, *n*=8-9, unpaired t-test,  $t_{(15)}=0.4623$ ; *p*=0,6505), CaMKII (APP<sup>swe</sup>/PS1<sup>dE9</sup>: 0.4811% ±13.26, *n*=8-9, unpaired t-test,  $t_{(15)}=0.032$ , *p*=0.9748) and synaptophysin (APP<sup>swe</sup>/PS1<sup>dE9</sup>: 2.78% ±6.03, *n*=8-9, unpaired t-test,  $t_{(15)}=0.3843$ , *p*=0.7061) were also unaffected. Interestingly, similar profiles were observed in human patients suffering from mild cognitive impairment, a pre stage of AD, by Sultana *et al.* (Sultana *et al.*, 2010).



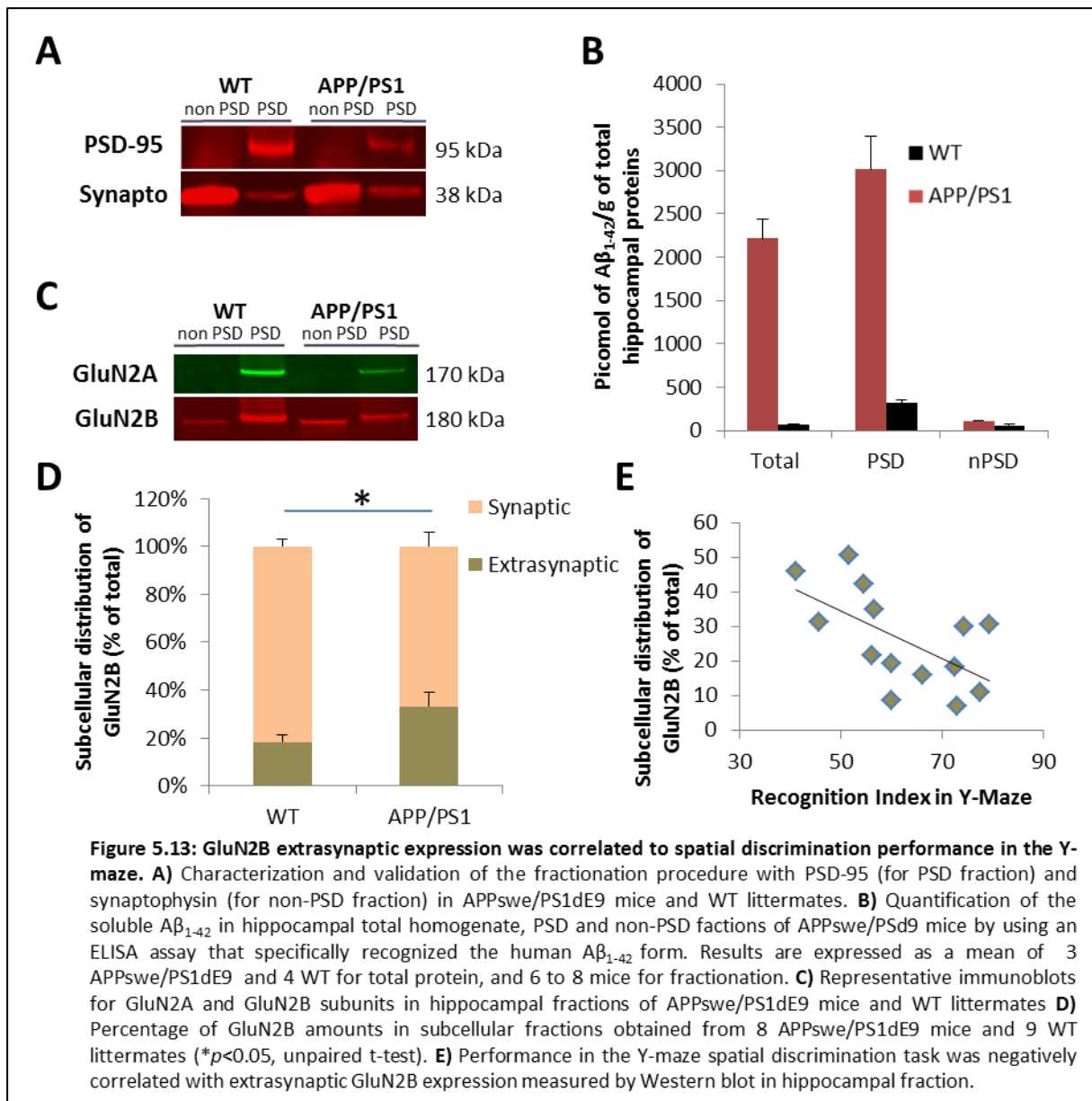
We next formally tested the relationship between the synaptic changes mentioned above and the spatial cognitive performance of APP<sup>swe</sup>/PS1<sup>dE9</sup> and WT mice. We focused on PSD-95 and GluN2A expressions which were altered in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice measured by Western blots.

Levels of these proteins were tested for a possible correlation with cognitive performance obtained in the Y-maze. Based on the key role played by PSD-95 and NMDAR in memory processing, we predicted a strong correlation between PSD-95 and/or GluN2A and cognitive performance. Linear regression analyses confirmed this prediction for PSD-95, ( $r=0.6092$ ,  $p=0.01$ ) (Figure 5.12) **but surprisingly not for GluN2A** ( $r=0.2654$ ,  $p=0.57$ ). As

expected, levels of expression of GluN2B, GluN1, synaptophysin or CaMKII, which were not significantly affected in our mutant mice, did not correlate with cognitive performance. Overall, our data thus supports studies suggesting a contributing role of PSD-95 in the physiopathology of AD.



It is well documented that PSD-95 is associated with NMDARs, acting as a scaffolding protein for these receptors at the synaptic site. Therefore, alterations of PSD-95 could compromise the trafficking and/or synaptic location of NMDARs. To investigate further possible changes in NMDAR location between the synaptic and extrasynaptic compartments, a fractionation protocol was applied as previously validated (Milnerwood et al., 2010). Using this protocol, we were able to isolate synaptic versus extrasynaptic pools of NMDARs from APPswe/PS1dE9 and age-matched WT hippocampi. We first characterized the fractionation protocol by checking the differential expression of PSD-95 and synaptophysin. As illustrated in Figure 5.13A, synaptic fractions were highly concentrated in PSD-95 whereas PSD-95 was not detectable in the non-synaptic fraction. In contrast, the presynaptic protein synaptophysin was highly concentrated in the non-synaptic fraction compared to the synaptic fraction (Figure 5.13A).



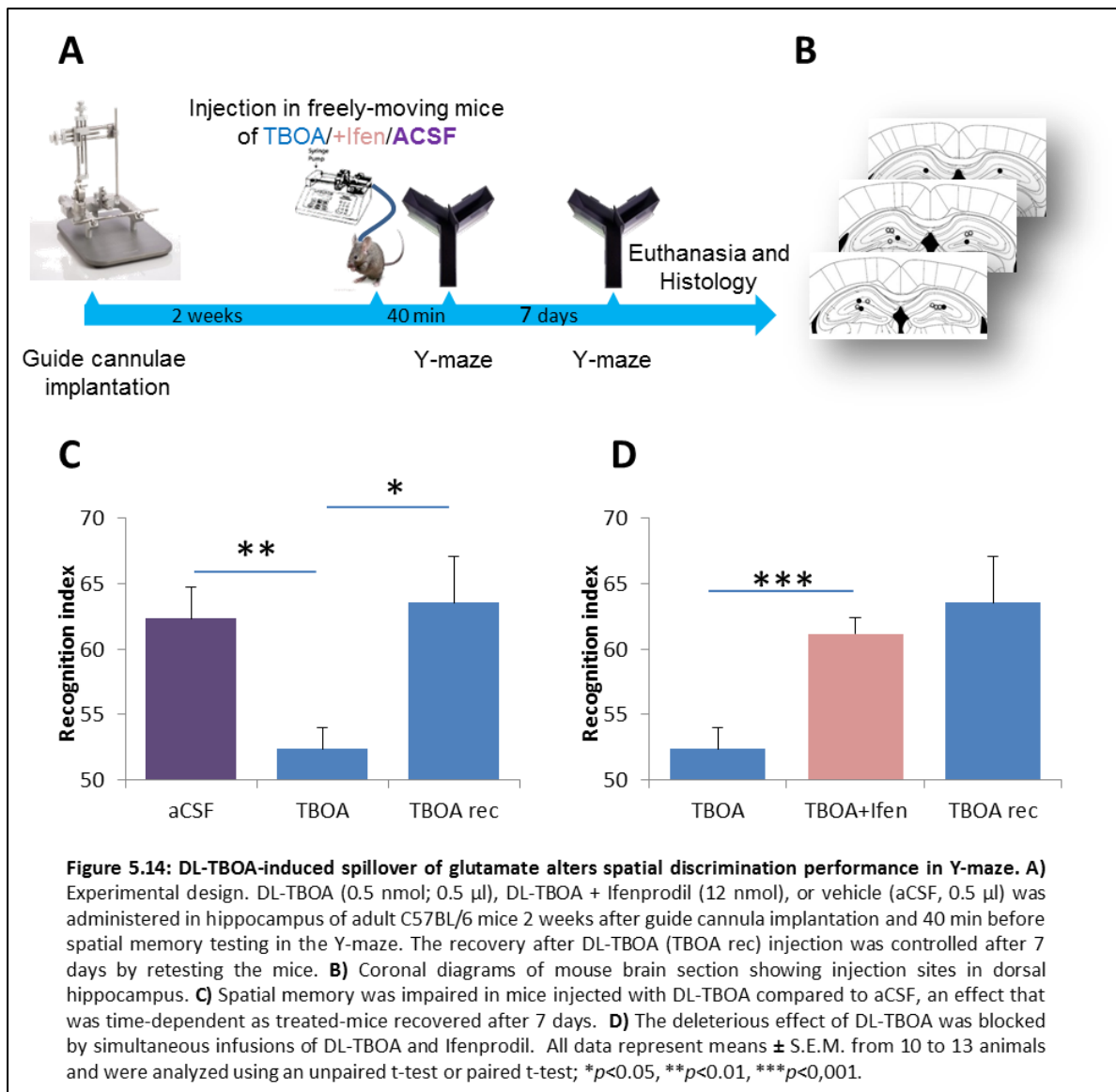
Because the post-synaptic density area is the main target of A $\beta_{1-42}$ , we quantified the A $\beta_{1-42}$  concentration in these fractions by using human A $\beta_{1-42}$  ELISA. As showed in Figure 5.13B, the soluble level of A $\beta_{1-42}$  was very low in the non-PSD fraction whereas the soluble A $\beta_{1-42}$  was concentrated in the PSD-95 positive fraction. Thus, these findings show that soluble A $\beta_{1-42}$  seems to specifically target the post-synaptic density fraction and this accumulation could interfere with synaptic receptor trafficking.

Next, we analyzed and quantified the expression of GluN2A and GluN2B in both synaptic and non-synaptic fraction. As expected, the synaptic fraction expressed both GluN2A and GluN2B subunits, but only GluN2B was detectable in the extrasynaptic location

confirming that the NMDAR containing GluN2A subunit was mainly located in the synaptic region whereas the GluN2B subunit was located at both synaptic and extrasynaptic sites as previously described (Hardingham and Bading, 2010). We also confirmed that the synaptic GluN2A expression was downregulated in APP<sup>swe</sup>/PS1dE9 mice (APP<sup>swe</sup>/PS1dE9:  $-33.87\% \pm 14.45$ , unpaired t-test  $t_{(13)}=1.923$ ,  $p=0.0767$ ) compared to age-matched WT mice (WT:  $0\% \pm 5.03$ ). We next analyzed the expression of GluN2B in both synaptic and extrasynaptic fractions but also by adding synaptic and extrasynaptic values to obtain total expression.

Interestingly, as shown in Figure 5.11, we confirmed that the total of synaptic plus extrasynaptic GluN2B expression (arbitrary unit) was not different between APP<sup>swe</sup>/PS1dE9 ( $67.8 \pm 10.38$ ) and age-matched WT mice ( $84.99 \pm 7.72$ ) (unpaired t-test,  $t_{(13)}=1.35$ ,  $p=0.2$ ). We next analyzed the proportion of synaptic and extrasynaptic GluN2B for each mouse and we clearly found a decrease of synaptic and an increase of extrasynaptic GluN2B in APP<sup>swe</sup>/PS1dE9 mice compared to age-matched WT (unpaired t-test,  $t_{(13)}=2.409$ ,  $p=0.0316$ ) (Figure 5.13D). Based on the concept of a deleterious effect of extrasynaptic recruitment on cognitive performance, we predicted the existence of a strong negative correlation between extrasynaptic GluN2B level and cognitive performance. Linear regression analyses confirmed this prediction for extrasynaptic expression of GluN2B ( $r=0.5935$ ,  $p=0.00252$ ) (Figure 5.13E). Altogether this data suggest that accumulation of synaptic A $\beta$  could directly or indirectly increase extrasynaptic GluN2B activity and interfere with memory processes.

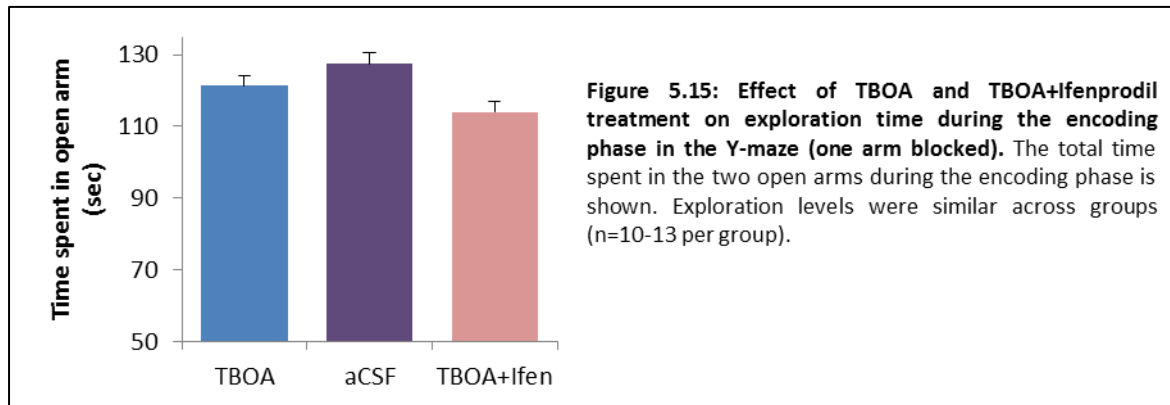
To further establish the validity of this hypothesis, we took advantage of the functional properties of the glutamate uptake inhibitor DL-TBOA which induces a spillover of glutamate and consequently, leads to the additional recruitment of extrasynaptic NMDARs. This strategy has been previously validated *in vitro* using brain slices (Kervern et al., 2012) but also *in vivo* (Han et al., 2012) to activate extrasynaptic receptors. We bilaterally injected DL-TBOA (0.5 nmol; 0.5 $\mu$ l) into the hippocampus (Figure 5.14B) of adult C57BL/6J mice 40 minutes before the encoding phase in the Y-maze. As expected, after a single encoding phase of 5 min with only two arms accessible, a short inter-trial interval of 10 min resulted in a robust preference for the unexplored (previously closed) arm during the test phase (Figure 5.14C) in aCSF-injected mice. In contrast DL-TBOA-injected mice failed to discriminate the novel arm (aCSF:  $62.33 \pm 2.354$ ,  $n=13$ ; DL-TBOA:  $52.34 \pm 1.614$ ,  $n=12$ ; unpaired t-test,  $t_{(23)}=3.445$ ,  $p=0.0022$ ).



To exclude the possibility that DL-TBOA induced irreversible excitotoxic lesions, DL-TBOA-injected mice were re-tested 7 days later. As illustrated in figure 5.14C, the previously DL-TBOA-treated mice could learn normally as shown by their ability to discriminate the new arm as well as the aCSF-injected mice (paired t-test:  $t_{(11)}=2.7$ ,  $p=0.0179$ ). Thus, this indicates that the observed DL-TBOA deleterious effect on memory performance was mainly related to a time-limited alteration of synaptic transmission rather than a nonspecific irreversible neuronal death resulting from DL-TBOA-induced excitotoxicity.

To evaluate the functional contribution of GluN2B subunits in the DL-TBOA-induced memory impairment, we performed bilateral injections of DL-TBOA and Ifenprodil (12 nmol), a selective GluN2B antagonist, into the hippocampus of C57BL/6J mice 40 min before the encoding phase. Only mice co-injected with Ifenprodil were able to discriminate the novel

arm, suggesting that ifenprodil blocked the synaptic alteration induced by the spillover of glutamate triggered by DL-TBOA (DL-TBOA + Ifenprodil:  $61.16 \pm 1.213$ ; DL-TBOA:  $52.34 \pm 1.614$ ; unpaired t-test:  $t_{(20)}=4.223$ ,  $p=0.0004$ ) (Figure 5.14D). This treatment-dependent impairment was memory-specific as there was no confounding effect of the treatment on the total exploration of the open arm during the encoding phase (Figure 5.15).



Altogether our data suggest that the synaptic accumulation of A $\beta$  could reduce the trafficking of GluN2B receptors at the synaptic area (inside the synapse), thereby increasing the presence of GluN2B in the extrasynaptic area of the synapse. Thus, this imbalance in the synaptic and extrasynaptic location of GluN2B subunits leading to an increased extrasynaptic GluN2B activity would be responsible, at least in part, of the altered memory profile of our transgenic AD mice.

### 3. DISCUSSION

By using proteomic analysis of whole hippocampus and specific neuronal compartments, we established that the cognitive performance of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice was correlated with the hippocampal expression of PSD-95. We also demonstrated that NMDAR expression was also altered in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice. Indeed, we evidenced that the downregulation of PSD-95 and/or synaptic accumulation of A $\beta_{1-42}$  were associated to a large reduction of GluN2A subunits but also to a mis-localization of the GluN2B subunits in the extrasynaptic compartment, which was inversely correlated to the cognitive performance of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice. By using an invasive approach and intracerebral injection of DL-TBOA, we were able to show that the extrasynaptic NMDAR activation triggered a reversible cognitive deficit in Y-maze, mainly related to GluN2B-containing NMDAR. Thus, our results suggest that the A $\beta$  destabilizes the synaptic organization and increases the extrasynaptic

pool of GluN2B-containing NMDARs, receptor reorganization which translates into impaired memory functions.

Loss of synapses at a fine structural level as well as reduction in synaptic markers have been well documented in early and late stages of AD (Davies et al., 1987; Masliah et al., 1991; Dickson et al., 1995; Sze et al., 1997; Masliah et al., 2001) and have been shown to correlate well with the extent of cognitive deficits (DeKosky and Scheff, 1990; Terry et al., 1991). Whereas most of these studies have used presynaptic markers such as synaptophysin, postsynaptic changes have not been extensively investigated and the reported changes of PSD-95 are still controversial. Indeed, a study measuring the expression of PSD-95 found a significant decrease in AD temporal cortex but not in less affected occipital cortex (Proctor et al., 2010). This downregulation of PSD-95 protein has also been reported in hippocampi (Sultana et al., 2010) and in synaptosomes prepared from cerebral cortex (Gyls et al., 2004) from AD patients. In contrast, studies using immunohistochemistry showed an increase in the postsynaptic marker PSD-95 with a decrease in the presynaptic marker synaptophysin in the cerebral cortex and hippocampus in AD brains (Leuba et al., 2008a; Leuba et al., 2008b). By using transgenic mice models of AD, studies have confirmed that PSD-95 is reduced in hippocampus and more especially in hippocampal apical dendrites (Shao et al., 2011). In our study we confirmed this downregulation of PSD-95 in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice and we also clearly showed a correlation between PSD-95 loss and cognitive deficit in AD mice in line with the observation that PSD-95 knock-out mice showed impaired learning abilities (Migaud et al., 1998). Thus PSD-95 could serve as a molecular marker for behavioral deficit. It will be interesting to know if the changes of PSD-95 can be prevented or even restored with various therapeutic treatments.

However, PSD-95 is not only a structural protein but also a key regulator of NMDAR trafficking (Bard and Groc, 2011). The loss of PSD-95 in the early phase of AD could first alter the synaptic receptor function(s) and later on the synaptic structure. In this line, we demonstrated that the expression of NMDAR subunits was altered in their expression levels and their subcellular locations. Indeed, as previously observed in human patient with mild cognitive impairment (Sultana et al., 2010), we found a downregulation not only of PSD-95, but also of the synaptic GluN2A. In contrast, the total expression of GluN2B was not affected in our mice, but interestingly we found a change of GluN2B subcellular distribution in favor



to extrasynaptic compartment. Thus, the alteration of GluN2 expression and/or subcellular distribution could be the consequence of PSD-95 downregulation. Indeed, the interaction between PSD-95 and GluN2 subunits of NMDARs has been shown to traffic the NMDARs to synapses from ER (Kornau et al., 1995; Sans et al., 2003; Cousins et al., 2008) and to regulate cell surface expression via inhibition of receptor internalization (Lavezzari et al., 2004). But how can we explain the difference between GluN2A and GluN2B? One possible explanation could be in a difference in cellular kinetics of trafficking, induced by different cytoplasmic carboxyl-terminal domain internalization motifs (Lavezzari et al., 2004; Groc et al., 2006). This difference in internalization motifs led to higher mobility, lateral diffusion, faster internalization, endocytosis and recycling of GluN2B-containing NMDARs, and slower internalization and more fusion with degradative endosomes of GluN2A-containing NMDARs (Lavezzari et al., 2004; Scott et al., 2004; Prybylowski et al., 2005). Because less recyclable, the GluN2A expression could be more sensitive to the reduction of PSD-95 than GluN2B.

Another main consequence of this GluN2A downregulation is the change in GluN2A/GluN2B ratio. Accordingly, this ratio plays a critical role in several neuronal processes involved in memory formation as the spine motility and synaptogenesis (Gambrill and Barria, 2011), the regulation of synaptic plasticity threshold (Lee et al., 2010a) and more generally in the synapse stability. This pathological switch is also functionally important because GluN2B-containing NMDARs have a longer decay time constants (Cull-Candy and Leszkiewicz, 2004) and carry more  $Ca^{2+}$ , which can initiate oxidative stress and neuronal death (Mehta et al., 2012).

Interestingly, we confirmed the decrease of synaptic GluN2B as observed by several groups (Snyder et al., 2005; Li et al., 2011) but in contrast we also found an increase of extrasynaptic expression of GluN2B, which could be due to a redistribution of GluN2B receptors, since the total expression of GluN2B was not affected. Snyder and colleagues were unable to detect a difference in the extrasynaptic staining of NMDARs in response to  $A\beta$  (Snyder et al., 2005), suggesting that the  $A\beta$ -induced NMDAR endocytosis could be specific to synaptic NMDARs, not to extrasynaptic receptors as also observed by Li et al. (2011). This difference could be related to pathological process mimicked by the model: early stage for exogenous  $A\beta$  application or late stage for our 9 month-old APP<sup>swe</sup>/PS1<sup>dE9</sup>. A timecourse of GluN2B changes in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice may solve this issue. However, we

and others highlight the alteration of the balance between synaptic and extrasynaptic NMDAR signalling in AD.

Until now, the majority of studies have characterized the role of extrasynaptic receptors in synaptic plasticity using both LTP and LTD model in hippocampal slices. As observed for exogenous application of A $\beta$  (Shankar et al., 2008), the functional recruitment of extrasynaptic NMDARs, blocks the development of the LTP and induces or potentiates the LTD process (Potier et al., 2010; Han et al., 2012). In our study, we took advantage of cerebral injection of DL-TBOA, to mimic the imbalance between synaptic and extrasynaptic receptor response. We demonstrated that the recruitment of extrasynaptic response altered the cognitive performance of mice, by a GluN2B-dependent mechanism. Altogether, these data suggest that mislocation of GluN2B receptors and/or the imbalance between synaptic and extrasynaptic response could disturb the encoding of new memory in AD. This last point is supported by several studies that show a beneficial effect of GluN2B antagonist to counteract the A $\beta$  effect (Klyubin et al., 2005). Our study could also explain the beneficial effect of memantine, one of the first drugs that were approved to alleviate symptoms in the middle and later stages of AD, and that selectively blocked the extrasynaptic NMDAR (Leveille et al., 2008; Xia et al., 2010). This increase of extrasynaptic response has been recently suggested. We found that this increase could be due to a redistribution of NMDARs, while others studies suggested that the recruitment of extrasynaptic NMDAR in AD could be due to a glutamate spillover, related to glial transporter dysfunctions (Li et al., 2009).

Although our results did not identify the mechanisms by which A $\beta$  disrupt PSD-95 and/or NMDAR location, we observed a synaptic accumulation of A $\beta$  in PSD but not in non-PSD fraction. This data confirmed the *in vitro* studies (Deshpande et al., 2009 ; Lacor et al., 2007) and were also in line with observations in human patients. Indeed, dimeric A $\beta$  oligomers in synaptosomes has been inversely associated with Mini Mental State Examination scores (Williams, 2009), and recently both monomeric and dimeric A $\beta$  species from soluble and detergent extracts have been strongly associated with dementia in AD cases (Mc Donald et al., 2010). Several neuronal receptors have been proposed as binding partners of A $\beta$ , including the  $\alpha$ -7-nicotinic (Snyder et al., 2005), glutamatergic (De Felice et al., 2008; Irvine et al., 2008 ; Renner et al., 2010; Zhao et al., 2010), insulin (De Felice et al., 2009) or cellular prion protein (Laurén et al., 2009) receptors. Most of them have been also

described in the postsynaptic density and could explain the preferential targeting of A $\beta$ . Also, these receptors reside preferentially within cholesterol-rich microdomains within the plasma membrane known as lipid rafts which play a key role in A $\beta$  production and aggregation (Rushworth and Hooper, 2010). The aberrant accumulation of A $\beta$  in this lipid raft could directly alter the mobility of lipid raft proteins such as glutamate receptors as previously observed for mGluR5 (Renner et al., 2010). Thus A $\beta$  oligomers accumulation in excitatory synapses may represent an initiating factor in AD pathology.

## **C. Absence of learning-induced increase in hippocampal ripple rate is associated with impaired spatial memory consolidation in a mouse model of Alzheimer's disease**

### **1. INTRODUCTION**

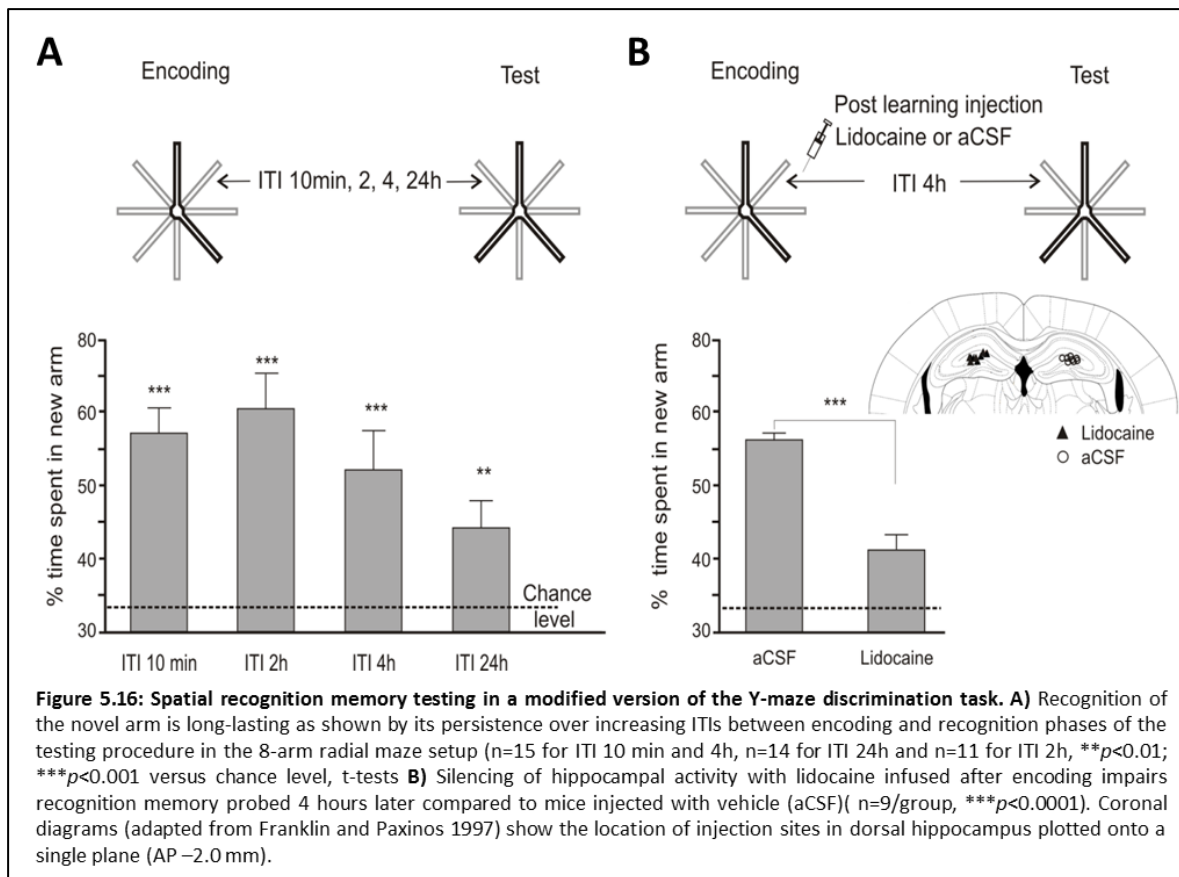
Information processing and memory formation in rodents have been reported to be accompanied by an array of hippocampal field potential oscillations that are important functionally. For instance, theta oscillations occur during active behavior and rapid eye movement (REM) sleep and have been suggested to provide the temporal frame for the encoding of information (Buzsaki and Watson, 2012). Gamma oscillations triggered during exploratory behavior are thought to be involved in memory acquisition (Axmacher et al., 2006) and their synchronization contributes to successful execution of working memory (Yamamoto et al., 2014). During slow wave sleep (SWS) that follows learning, hippocampal circuits consistently increase the occurrence rates of sharp wave-ripples (SWRs) which typically recur at 0.4 to 1 Hz (Cheng and Frank, 2008; Eschenko et al., 2008). Importantly, upon occurrence of SWRs, ensembles of hippocampal place cells can replay in faster timescales their sequential activity triggered during a previous learning episode, suggesting an essential role for SWRs in driving memory consolidation processes and subsequent long-term stabilization of newly acquired spatial memory traces (Girardeau and Zugaro, 2011). When such SWRs are experimentally disrupted, it causes memory deficits in hippocampus-dependent memory tasks (Girardeau et al., 2009), further suggesting that abnormal hippocampal rhythmic activity can interfere with hippocampal information processing, a dysfunctional pattern also observed in pathological conditions such as AD (Schreiter-Gasser et al., 1994).

The cognitive impairments associated with AD are related to degenerative synaptic changes produced by the presence of soluble amyloid beta proteins (A $\beta$ s) in vulnerable brain regions such as the hippocampus considered to be critical for spatial learning and declarative memory (Frankland and Bontempi, 2005). There is increasing evidence that early oligomeric forms of A $\beta$ s, rather than late fibrillar conformations, interfere with neuronal network functional properties and are responsible for cognitive dysfunctions in AD patients (Lue et al., 1999) as well as in transgenic mouse models of this disease (Mucke et al., 2000). It has been found that A $\beta$  oligomers (A $\beta$ os) differentially affects hippocampal network activities,

reducing theta and gamma oscillations *in vitro* (Driver et al., 2007) while surprisingly sparing SWRs (Hermann et al., 2009). Close examination reveals that such a lack of effect may be the consequence of recording hippocampal activity either in cell cultures or in animals remaining in their home cage, a basal condition which may hinder an effect of A $\beta$ os on SWRs otherwise detectable in cognitively-challenged animals. Here, we sought to unravel the action of A $\beta$ os on neuronal populations involved in the generation of SWRs in mice undergoing encoding and consolidation of spatial information. To this end, we submitted mice to spatial recognition memory testing in a modified version of the Y-maze discrimination task tailored to maximizing spatial cognitive demand. After confirming the hippocampal-dependency of this task, we established its ability to detect spatial memory impairments after intracerebroventricular infusion of A $\beta$ os. We then determined the signature of this A $\beta$ o treatment on hippocampal SWRs in mice without cognitive requirements or while undergoing a single spatial discrimination session.

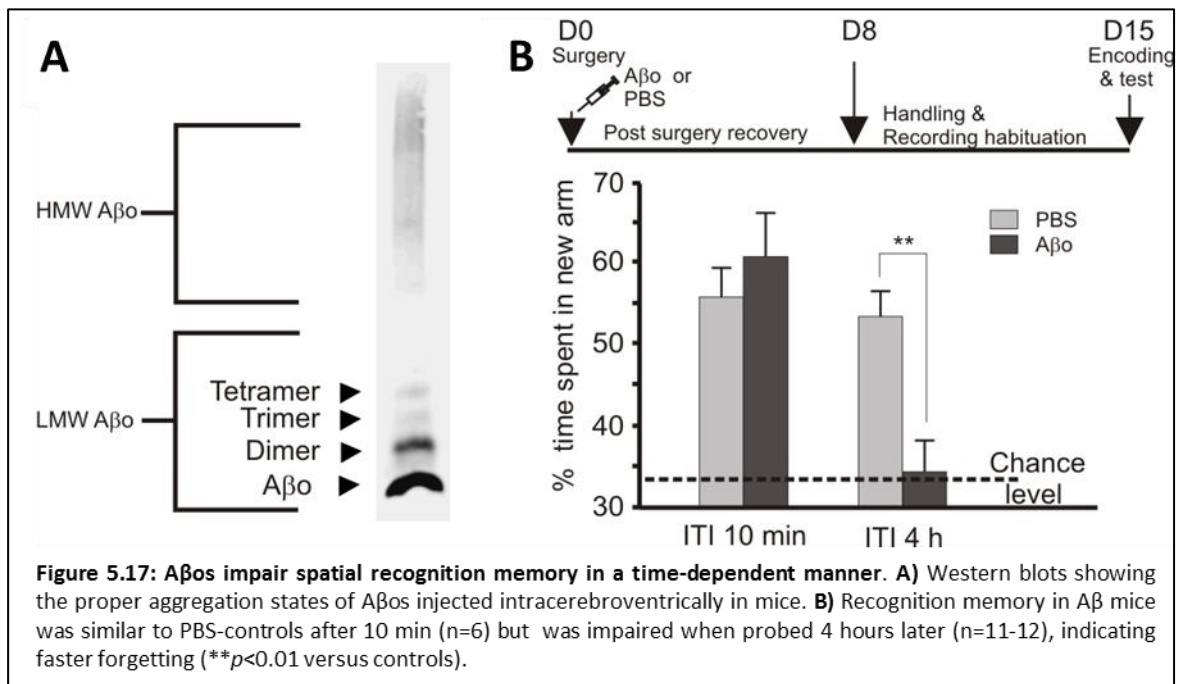
## 2. RESULTS

To characterize the effects of A $\beta$ os on spatial recognition memory and training-induced hippocampal ripples, we used a modified version of the Y-maze two-trial arm discrimination task conducted in an 8-arm radial maze apparatus and designed to increasing spatial cognitive demand (Figure 5.16 and Discussion). As expected, after a single encoding phase of 10 min with only two arms accessible, a short ITI of 10 min resulted in a robust preference for the unexplored (previously closed) arm during the test phase (Figure 5.16). Interestingly, increasing the ITI from 10 min to 24 hours revealed spatial recognition memory performance that was still above chance within this extensive time window (Figure 5.16). Importantly, bilateral region-specific inactivation of the hippocampus with the sodium channel blocker lidocaine infused immediately after encoding impaired recognition memory probed 4 hours later ( $n=9/\text{group}$ ,  $t_{16}=5.85$ ,  $p<0.0001$ ), thus confirming the supportive role of the hippocampus in the formation and expression of recognition memory (Figure 5.16).

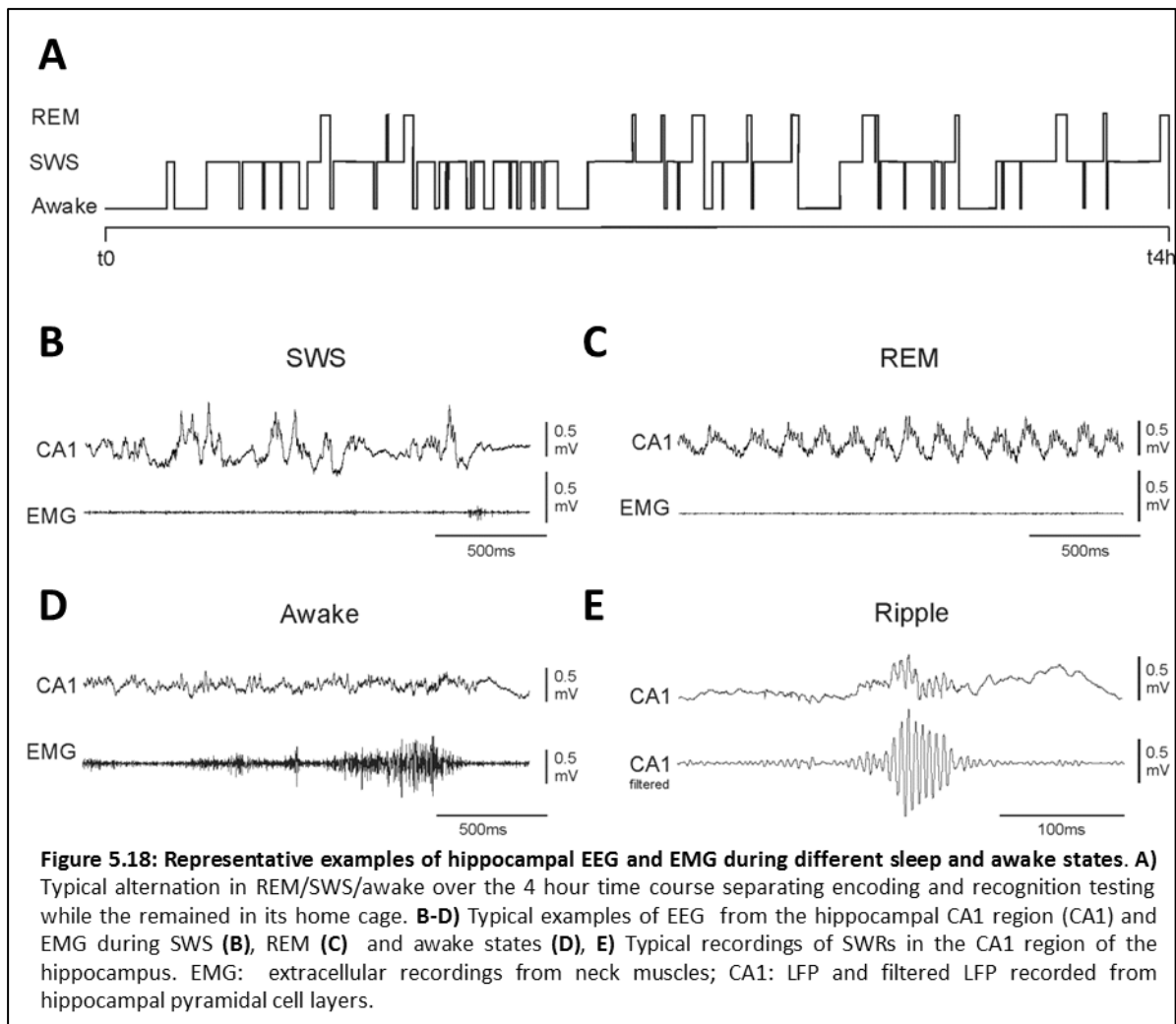


We next sought to unravel the impact of A $\beta$ os on memory performance. Fifteen days following a single intracerebroventricular injection of a mixture of proper low and high molecular weights of A $\beta$ os or vehicle (PBS) (Figure 5.17), we examined recognition memory performance either 10 minutes or 4 hours after encoding (Figure 5.17). Following the short retention delay, both PBS- and A $\beta$ o-injected mice spent more time in the novel arm compared to the familiar (previously visited) ones (Figure 5.17; n=6/group). In contrast, when the retention delay between the encoding and the test phases was increased, A $\beta$ o-injected mice failed to discriminate the novel arm. They performed at chance while control mice were still successful and exhibited a performance level similar to that observed after the short retention delay (Figure 5.17). This delay-dependent A $\beta$ o impairment was memory-specific as there was no confounding effect of the A $\beta$  treatment on the total exploration time during either the encoding phase (A $\beta$  mice: 222.49 sec  $\pm$  29.36; PBS mice: 218.09 sec  $\pm$  23.25,  $t_{(21)}=0.12$ , NS, n=11-12) or the test phase (A $\beta$  mice: 105.06 sec  $\pm$  23.24; PBS mice : 131.36 sec  $\pm$  26.74,  $t_{(21)}=0.74$ , NS). Likewise, there was no preference for a particular arm (arm preference, two-way ANOVA,  $F_{(1, 42)}=0.0029$ , NS) and no effect of treatment in arm preference (Interaction: arm preference X treatment; two-way ANOVA,  $F_{(1, 42)}=0.2415$ , NS)

during the encoding phase (open arm 1: A $\beta$  mice: 108.21 sec  $\pm$  13.69; PBS mice 112.82 sec  $\pm$  13.34; open arm 2: A $\beta$  mice: 114.27 sec  $\pm$  16.6; PBS mice: 105.27 sec  $\pm$  11.69, n=11-12). Collectively, these findings indicate that A $\beta$ -injected mice were capable of processing visuo-spatial information and forming short-term recognition memory. However, when the retention delay was extended, they exhibited accelerated forgetting, a memory profile also observed in transgenic mouse models of AD (Daumas et al., 2008).

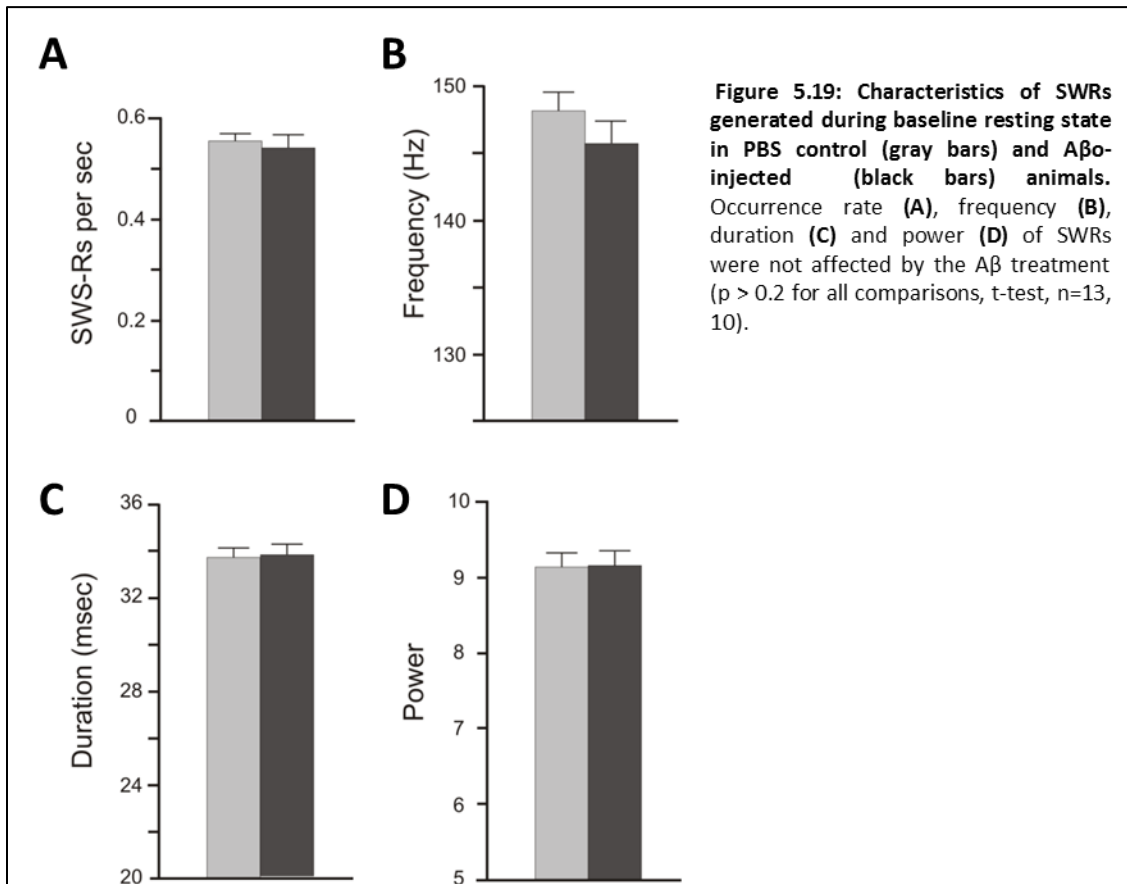


The memory profile of A $\beta$ -injected mice points to an inability to form a stable memory over time and is suggestive of impaired consolidation processes during which SWRs are thought to play a privileged role (Girardeau and Zugaro, 2011). To examine the effects of A $\beta$ os on the dynamics of SWRs, we recorded the extracellular field potential activity in the CA1 region which enabled us to pinpoint and characterize different sleep/awake stages triggered in our recognition memory paradigm. A typical example of a SWS/REM/awake alternation as well as corresponding EMG and EEG patterns of each of these states are illustrated in Figure 5.18. We focused our analysis only on SWRs occurring during the SWS bouts.



When analyzing the characteristics of SWRs (baseline occurrence rate, frequency, duration and power) during 80 min of quiet resting state in home cage, we found that the overall SWRs properties were left unaffected by the A $\beta$  treatment (Figure 5.19A-D). The occurrence rate, frequency, duration and power of SWRs were very similar between PBS- and A $\beta$ -injected groups (NS for all comparisons, t-test, n=10-13). This finding is in agreement with previous observations showing no alteration of ripples properties by A $\beta$  (Hermann et al., 2009). In sharp contrast, cognitively-challenged A $\beta$  mice exhibited impaired SWR patterns compared to PBS-control mice (see below).





To examine the effects of A $\beta$ os on SWRs occurrence as a function of the cognitive demand, we recorded the extracellular field potential activity in the CA1 region of the hippocampus over 10 time intervals of 40 minutes distributed as follows: baseline activity prior to memory encoding (2 intervals), encoding-induced activity (6 intervals) and testing-induced activity (2 intervals) (Figure 5.20, top panels). This segmented time course enabled us to pinpoint and characterize the dynamics of SWRs occurring in resting conditions and at different stages of spatial memory processing, namely encoding, consolidation and recognition.

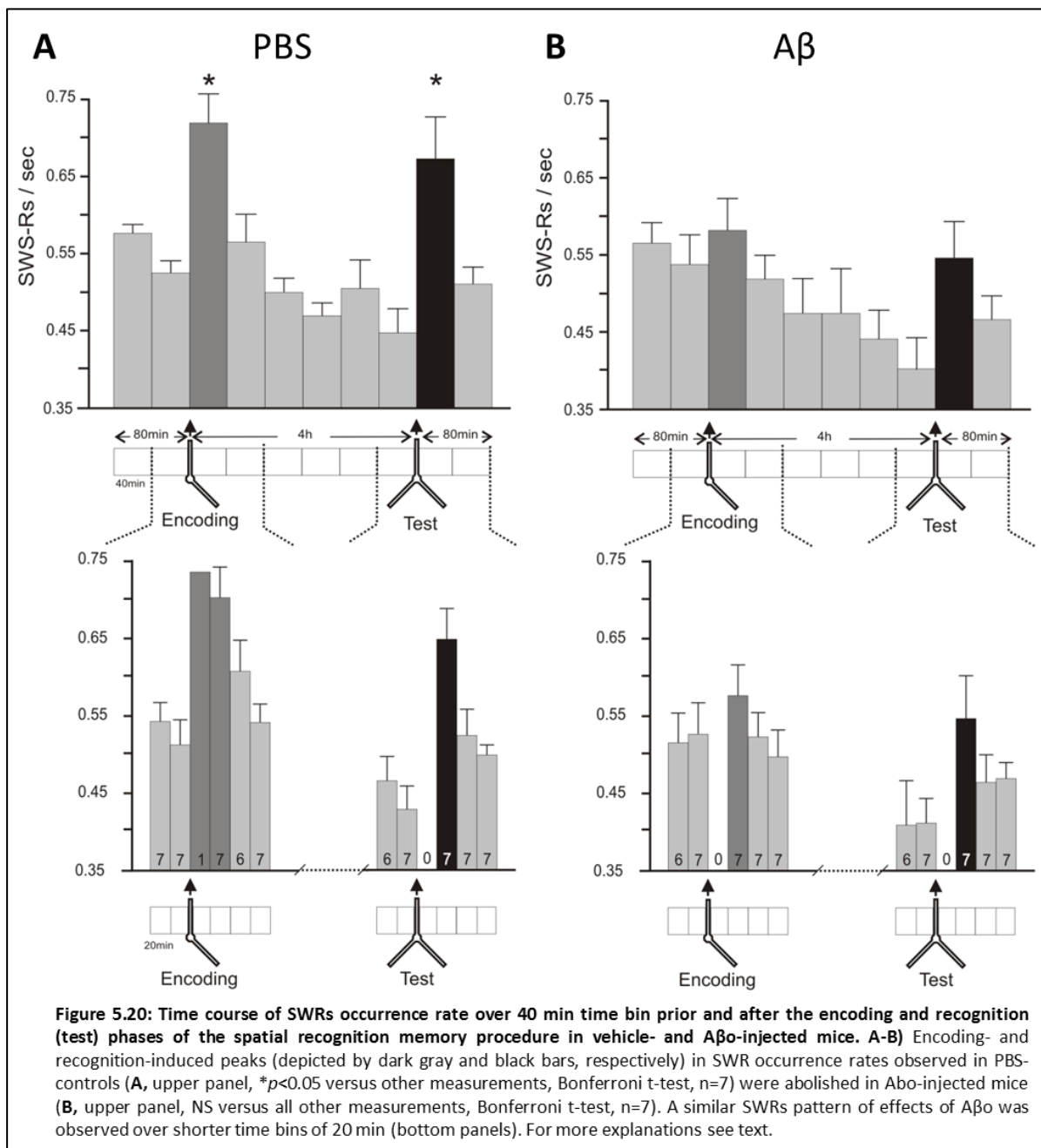
It must be noted that the occurrence rate and duration of SWS episodes over the time course of the experiment were similar in the two groups (occurrence rate: A $\beta$  mice 7.1 per hour  $\pm$  0.61; PBS mice, 8.01 per hour  $\pm$  0.73; duration: A $\beta$  mice, 5.36 min  $\pm$  0.5; PBS mice, 4.68 min  $\pm$  0.49;  $F < 1$ , NS for all comparisons,  $n=7$ /per group). REM episodes were also similar in both groups (occurrence rate: A $\beta$  mice 3.89 per hour  $\pm$  0.14, PBS mice 3.75 per hour  $\pm$  0.19; duration: A $\beta$  mice 1.20 min  $\pm$  0.15; PBS mice 1.23 min  $\pm$  0.15;  $F < 1$ , NS).

Also, the amount of SWS per 40 min bin was similar in both groups and ranged from 22.32 min  $\pm$  3.53 to 30.41 min  $\pm$  1.5 in PBS and from 23.15 min  $\pm$  3.56 to 33.46 min  $\pm$  1.02 in A $\beta$ -injected animals with the exception of the post-encoding and post-test periods (bins 3 and 9, in which first 20 min was usually occupied by awake state). During these specific time bins the amount of SWS ranged from 12.79 min  $\pm$  1.76 to 12.97 min  $\pm$  1.85 in PBS and from 12.24 min  $\pm$  2.05 to 14.22 min  $\pm$  1.95 to in A $\beta$ -injected group.

Interestingly, during the course of this experiment, two peaks of hippocampal SWRs occurrence were clearly apparent in PBS-control mice, one triggered upon exploration of the two available arms of the 8-arm radial maze, the other occurring upon the recognition phase of the testing procedure during which mice successfully identified the presence of the new open arm (see stars, upper panel, Figure 5.20A). Indeed, ANOVA revealed a significant main effect of “time bins” in the PBS group ( $F_{(6,9)}=16.16$ ,  $p<0.0001$ ) which was due to an increase of post-learning ( $p<0.05$  versus all other measurement, t-test) and post-test occurrence of SWRs ( $p<0.05$  versus all measurements except first bin and post-learning bin, t-test). These findings reveal that a single learning session is sufficient to produce an increase in the hippocampal SWR occurrence rate, thereby reflecting an important involvement of hippocampal oscillations in memory formation. However, contrasting with memory paradigms involving multiple training sessions (Eschenko et al., 2008), no significant changes in power, duration or frequency of SWRs were observed either after encoding or recognition testing ( $F<1$ , NS for all comparisons,  $n=7$ , data not shown).

In sharp contrast, cognitively-challenged A $\beta$  mice exhibited impaired SWR patterns compared to PBS-control mice. Namely, the encoding and recognition-induced peaks in the SWR occurrence rate observed in the control group (Figure 5.20A, upper panel) were abolished in A $\beta$  animals (Figure 5.20B, upper panel). Indeed, ANOVA with “time bins” as repeated measurements and “treatment” (A $\beta$  vs PBS) as between-subjects variable showed significant effect of “time bins” ( $F_{(12,9)}=24.02$ ,  $p<0.0001$ ) as well as “time bins” x “treatment” interaction ( $F_{(12,9)}=3.12$ ,  $p=0.002$ ), indicating that the dynamic of SWRs during the course of experiment was different in the two groups of the animals. Finally, comparison of the incidence of SWRs during all time bins between control and A $\beta$ -injected animals revealed significant difference for the post-encoding period ( $t=2.48$ ,  $p=0.029$ ) and a difference close

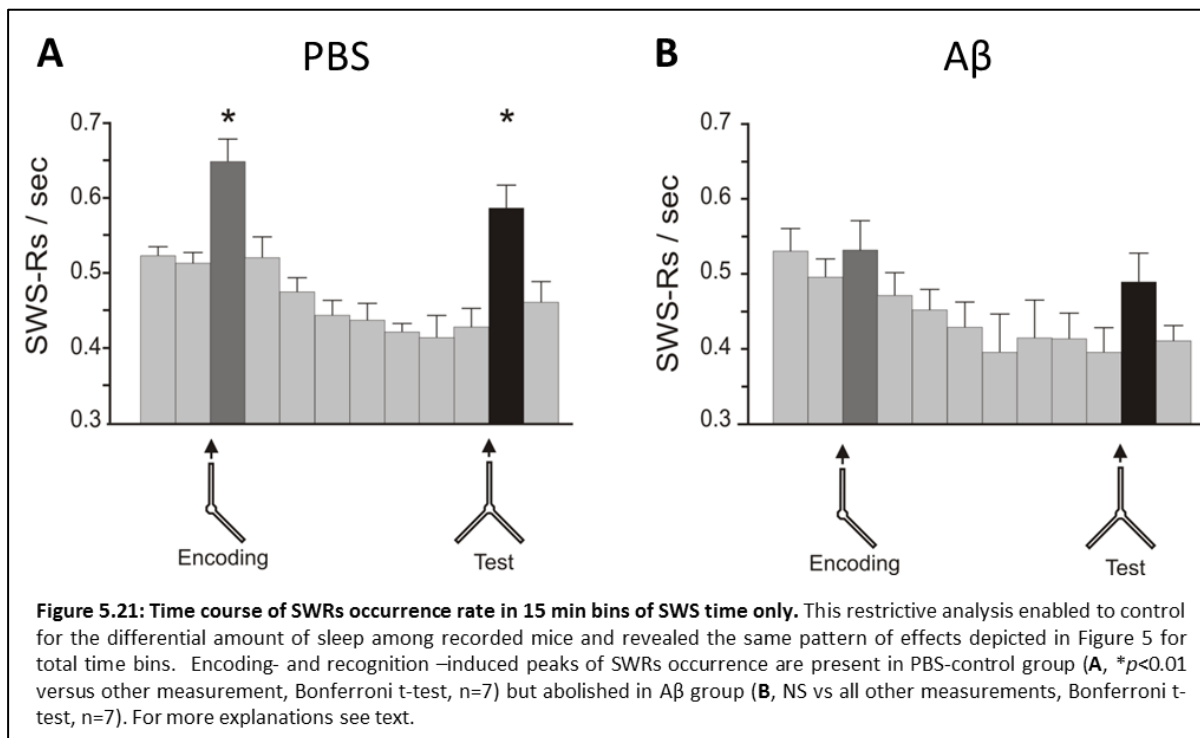
to significance for the post-test period ( $t=2.09$ ,  $p=0.058$ ) with all other measurements remaining similar between the two groups ( $p>0.2$ ).



When refining our analysis of SWR dynamics by restricting it to shorter time bins of 20 min, we found similar patterns of SWR occurrence in PBS-controls and A $\beta$ -injected mice (Figure 5A-B, bottom panels). Since accuracy of ripple rate estimation decreases for very short SWS bouts, we only took into account animals which expressed at least a 5 cumulated min of SWS in the bin. This resulted in unequal animal numbers in the 20 min bins (see numbers within bars, bottom panels) and rendered impossible the use of an ANOVA similar

to that performed for 40 min bins histograms. However, a comparison of SWRs occurrence rate during 20 min bins confirmed a significant difference between PBS-control and A $\beta$  groups during the second post-encoding ( $t=2.43$ ,  $p=0.032$ ,  $n=7$ ) and second post-test 20 min bin ( $t=2.17$ ,  $p=0.05$ ,  $n=7$ ).

Binning total time and not SWS time might imply that for some animals the rate of SWRs for a first 40 min bin was calculated for instance over the first 5 min of SWS whereas for another time bin, it was calculated over the first 30 min, depending on how much the animal have slept during this time. Therefore we performed additional analysis taking into account time bins corresponding to SWS only (Figure 5.21).



To better control for this potential confound, we chose to express the time course of SWR occurrence rate in 15 min bins of SWS time. These 15 min SWS bins were calculated separately for 3 parts of the experiment, starting from the first SWS bound that the animal expressed within each part: baseline recording (bins 1-2), between encoding and test (bins 3-10) and after test (bins 11-12). For each part the number of SWS bins was truncated to the minimal amount of SWS that all mice of the group have slept.

This analysis confirmed the abolishment of learning-induced increase of SWRs occurrence rate in A $\beta$ -injected animals. Indeed, besides the main effect of repetition ("SWS

bins”) ( $F_{(12,11)}=22.56$ ,  $p<0.0001$ ) a two- way ANOVA showed significant “SWS bins” x “treatment” interaction ( $F=2.33$ ,  $p=0.012$ ), indicating different time course of SWRs occurrence in the two groups. Moreover, a one-way ANOVA showed a significant main effect of repetition in the PBS-control group ( $F_{(6,11)}=15.1$ ,  $p<0.0001$ ) and A $\beta$ -injected animals ( $F_{(6,11)}=9.11$ ,  $p<0.0001$ ). In the control group the post-hoc analysis revealed a significant increase of post-learning and post-test occurrence of SWRs ( $p<0.05$  versus all other measurements, Bonferroni t-test). By contrast, in A $\beta$  animals no difference between occurrence rate in SWS bins was found (NS for all comparisons, Bonferroni t-test). Also direct comparisons of the SWRs occurrence rates in all SWS bins between two groups showed significant difference for the post-encoding period ( $t=2.41$ ,  $p=0.032$ ), a difference approaching significance for the post-test period ( $t=2.0$ ,  $p=0.068$ ) and no difference for all remaining bins ( $p>0.2$ ). Together, these results demonstrate that the deleterious effect of A $\beta$ os on the dynamics of SWRs is activity-dependent in nature and only effective in cognitively-demanding situations requiring hippocampal processing.

### 3. DISCUSSION

Recognition memory, a subdivision of episodic memory, is of particular interest in the context of AD as this form of memory is typically affected during the early stages of this neurodegenerative disease (Dubois et al., 2007). We adapted the classical two-trial recognition procedure in the Y-maze to the 8-arm radial maze in order to promote reliance on distal cues, thereby enhancing the spatial cognitive demand of the testing procedure. This adaptation highlighted the potential for a long-lasting spatial recognition memory which could last for at least 24 hours. Its hippocampal-dependent nature was confirmed by region-specific post-encoding inactivation of the hippocampus which impaired performance, thus pointing to the functional involvement of this brain region in supporting the formation and expression of spatial recognition memory.

Consistent with previous findings, our study reveals a transitory increase in the occurrence rate of hippocampal SWRs following a spatial learning episode, further strengthening the functional implication of SWRs in the progressive stabilization of spatial information during the course of memory consolidation processes (Girardeau et al., 2009). We identified two peaks in hippocampal SWR occurrence during the 40 min following either

the encoding or the recognition phases, a neuronal signature similar to that reported in associative spatial memory tasks in the rat (Eschenko et al., 2008; Ramadan et al., 2009). However, contrasting with an increase in ripple magnitude after new associative learning or long-term memory retrieval (Eschenko et al., 2008), we did not find any changes of SWR duration or power. This differential pattern may be due to the fact that in our recognition memory paradigm, mice were exposed only once to the maze prior to engaging into SWS when ripples were recorded whereas in the previous work, animals were subjected to intensive multiple training sessions in which they had to extract specific learning rules. Moreover, our testing procedure relied on the innate preference of rodents for novelty and did not involve any reward-associated learning.

Noteworthy is the transitory pattern of the two hippocampal SWR occurrence peaks observed upon encoding and recognition testing. They lasted only 40 minutes, a temporal dynamics which suggests that they may have acted primarily as a triggering switch during SWS for subsequent long-lasting cellular and molecular changes in weight and wiring plasticity within hippocampal cell assemblies actively engaged in processing the spatial layout of the maze environment. Another speculative proposition is that in a context of more ethological situation where animals must learn sequentially different information, it would be appropriate that these informations are processed as fast as possible (short peak of SWRs) to avoid overlapping. The ability of the animal to later recognize the maze environment requires the successful reinstatement of previously stabilized hippocampal place maps. SWRs are likely candidates for such a process of stabilization by strengthening spatial cell assemblies (Csicsvari and Dupret, 2014). Functionally, the encoding- and recognition-induced SWR drives we identified may convey different roles. Upon encoding, the hippocampal SWR occurrence peak could initiate the progressive stabilization during SWS of the general spatial configuration of the maze (i.e. access to two arms of the maze). Upon recognition testing, the SWR drive may reflect the partial remapping of hippocampal place fields related to the formation of an updated representation of the environment in which one additional arm of the maze is now available.

Because SWRs are triggered in cognitively challenged animals, their dysfunctional patterns are expected to impair memory-related processes. Accordingly, when disrupted experimentally, abnormal SWR signatures lead to impaired spatial learning (Ego-Stengel and

Wilson, 2010; Girardeau and Zugaro, 2011; Girardeau et al., 2014). With regards to neurodegenerative diseases such as AD, the functional contribution of SWRs to the reported impairments in spatial memory remains however poorly understood. To implement the observation that cognitive deficits of AD patients are correlated to soluble A $\beta$  levels rather than plaque-development *per se* (Lesne et al., 2013), we chose to inject intracerebroventrically synthetic forms of A $\beta$ os in mice. This model produces cognitive deficits much faster than other transgenic animal models in which memory impairments develop only within months and enables rigorous control over the time course of AD symptomatology (Chambon et al., 2011). We found that A $\beta$ o-injected mice exhibited faster forgetting compared to controls, a memory profile pointing to an inability to form and stabilize, or retrieve, long-lasting memories. Because the spatial recognition procedure relies on the natural tendency of animals to seek novelty, the possibility remains that the A $\beta$ o-treatment impacted other non-mnesic behavioral components, such as for instance reduced attraction to the novel arm or novelty-related increased anxiety that would prevent exploration of the novel arm during the testing phase despite remembering the previously explored arms. However, the observation of an intact recognition memory at a very short delay (10 minutes) in A $\beta$ o-injected mice makes these potential confounding factors unlikely. It further strengthens the existence of altered memory consolidation and retrieval processes, two mechanistic accounts already suggested in other transgenic models of AD in which only early state of A $\beta$  aggregation is present without plaque formation (Daumas et al., 2008). We found that the accelerated memory decay of A $\beta$ -treated mice was associated with an abolishment of the two time-limited peaks of SWRs normally seen in controls, suggesting that their occurrence likely constitutes a prerequisite for the formation and accurate expression of spatial recognition memory. At the mechanistic level, memory reactivation is considered as the core iterative mechanism in contemporary consolidation models. Hippocampal place cells that were co-active during spatial exploration exhibit correlated firing patterns during SWS, revealing a replay mechanism. Importantly, hippocampal replay retains the original temporal order, and occurs preferentially during the occurrence of SWRs (Frankland and Bontempi, 2005), thus conferring to these specific offline oscillations a privileged role in promoting weight and wiring synaptic plasticity and in coordinating memory consolidation across hippocampal-cortical networks. Importantly, our results demonstrate for the first time that it is a lack of post-learning increase in the SWR

occurrence rate, and not an absolute absence of SWRs (still generated normally in A $\beta$  mice prior to memory testing), which may be responsible for the impaired memory profile of A $\beta$  mice. This suggests no alteration of the neuronal mechanism underlying the generation of SWRs but points instead to its inability to respond adequately to a specific cognitive demand. This statement is further supported by a complete preservation of SWRs properties in A $\beta$ -treated mice in resting conditions, a finding which is also in agreement with the unaffected ongoing ripple activity demonstrated in slices from transgenic AD mice (Hermann et al., 2009) and rat A $\beta$ -treated slices (Adaya-Villanueva et al., 2010). Although many cellular and synaptic mechanisms can explain the A $\beta$ -induced lack of SWRs triggering upon a cognitive challenge, one putative candidate is NMDAR-induced synaptic plasticity. Indeed, it has been demonstrated that high level of A $\beta$ os can alter glutamatergic synaptic transmission which in turn can lead to synaptic loss (Mucke and Selkoe, 2012). Moreover, the post-learning increase drive for SWR occurrence has been recently proposed to result from NMDAR plasticity and early (upon encoding) neuronal tagging of hippocampal-cortical networks, a NMDAR-dependent neurobiological process required for the progressive embedding of memory traces within hippocampal-cortical networks during sleep and resting periods (Lesburgueres et al., 2011; Girardeau et al., 2014). It is therefore possible that the early A $\beta$ -induced alteration of NMDAR function may preclude the dynamic response of hippocampal networks to post-learning requirement.

In conclusion, our data provide novel insights into the functional involvement of SWRs in the spatial memory impairments observed in AD. While unaffected in basal conditions, the occurrence patterns of hippocampal SWRs associated with either encoding or expression of recognition memory were specifically disrupted in the event of a challenging situation. Because A $\beta$ -treated mice were able to form short-term but not long-term recognition memory, the absence of the SWR occurrence peak following encoding likely impacted predominantly consolidation processes involved in the subsequent stabilization of the hippocampal memory trace and not encoding processes *per se*. The failure in expressing long-term recognition memory of A $\beta$  mice was also associated with a lack of a dedicated SWR occurrence peak, possibly indicating that the memory has not been properly stabilized (faster forgetting) or that access to a partially degraded trace was no longer possible. While highlighting the crucial roles played by SWRs dynamics in hippocampal memory processing,

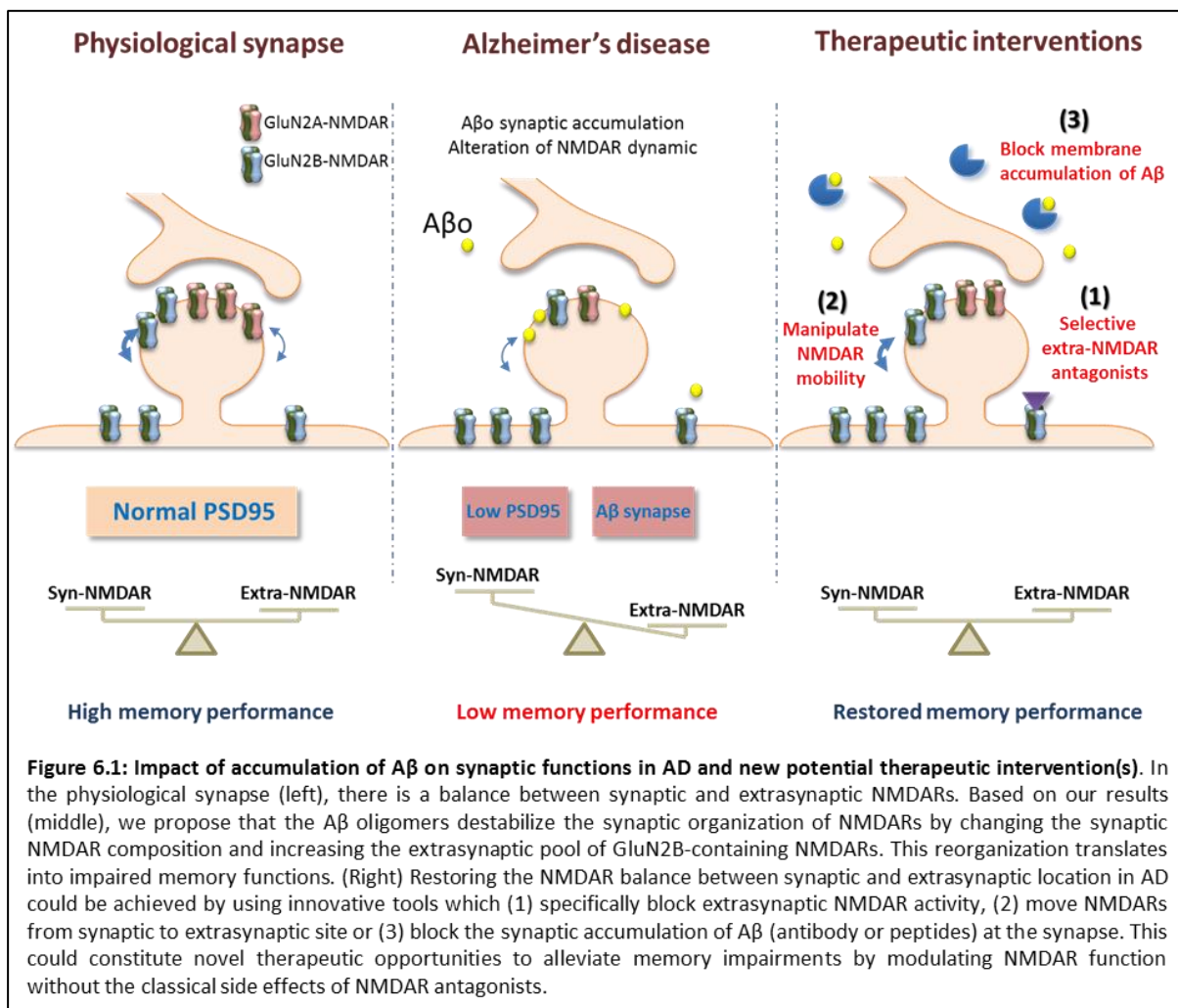


our findings also identified the absence of learning-induced SWR occurrence rates as a potentially early marker of AD.

## VI. GENERAL DISCUSSION

## A. SYNTHESIS OF RESULTS

AD is the most common cause of dementia characterized by progressive cognitive impairments which are related, in part, to the extracellular accumulation of A $\beta$ . Synaptic loss is an early and invariant feature of AD and there is a strong correlation between the extent of synaptic loss and the severity of dementia in human patients (Roth et al., 1966; Terry, 1996). At the molecular level, A $\beta$ s bind preferentially to the postsynaptic density of neuronal excitatory synapses, where the scaffolding proteins organize NMDAR composition and subcellular location (synaptic versus extrasynaptic). By using an integrative strategy favoring vertical levels of analyses (from phenotype to molecular events) and combining a set of interrelated correlative and invasive approaches in both normal mice and an animal model of AD (APPswe/PS1dE9), we established that the cognitive performance of APPswe/PS1dE9 mice was correlated with the hippocampal expression of PSD-95. The reduced expression of PSD-95 was associated to a mislocalization of the GluN2B subunit to the extrasynaptic compartment which was, further, inversely correlated to the cognitive performance of APPswe/PS1dE9 mice. Finally, we showed that the extrasynaptic NMDAR activation triggers a reversible cognitive deficit in the Y-Maze, mainly related to GluN2B-containing NMDARs. Altogether, our results showed that the A $\beta$  destabilized the synaptic organization of NMDARs, changed the synaptic NMDAR composition and increased the extrasynaptic pool of GluN2B-containing NMDARs, a reorganization which translates into impaired memory functions (Figure 6.1).



Several mechanisms could be responsible for the alteration of synaptic composition and location of NMDARs. First, the downregulation of PSD-95 could alter synaptic organization. Indeed, PSD-95 is a core component of the PSD of synapses and regulates the relative number and positioning of NMDAR subunits. Unraveling the mechanism underlying the PSD-95 reduction in AD could delay the onset or progression of cognitive deficits. Interestingly, several interventions have been proposed to increase the PSD-95 expression such as environmental enrichment which has been shown to delay the cognitive deficit in AD mice. In this context PSD-95 could be the molecular determinant of the cognitive reserve responsible for delaying or attenuating the cognitive impairment associated with AD.

The synaptic accumulation of Aβ could modify the synaptic membrane fluidity and consequently the NMDAR mobility and location. Indeed, NMDARs, as others synaptic receptors, reside preferentially within cholesterol-rich microdomains within the plasma membrane, known as lipid rafts. The aberrant accumulation of Aβ in this lipid raft could

directly alter the mobility of lipid raft proteins such as glutamate receptors as previously observed for mGluR5 (Renner et al., 2010). Thus, A $\beta$  oligomers accumulation in excitatory synapses may represent an initiating factor in AD pathology and blockade of this accumulation can have beneficial repercussion in cognitive alteration in AD.

The emerging possibility that NMDAR location is altered in pathological processes of AD has highlighted the need for an exploration of the impact of NMDAR trafficking in memory processes in normal mice. The set-in-stone dogma that NMDARs are stable in synapses has simply shattered over the last decade, opening a new conceptual framework to understand the glutamate synapse adaptations. Indeed, the complexity of synaptic NMDAR modulation has escalated with the knowledge that receptors can traffic between intracellular, synaptic and extrasynaptic sites. Electrophysiological, molecular and imaging approaches have demonstrated that the NMDAR composition is not only regulated by receptor cycling but also by the NMDAR surface diffusion. The tools to specifically modulate NMDAR trafficking have proven difficult to generate but the recent advances in the understanding of the dynamics and subcellular localization of NMDARs have fostered avenues for specific and efficient tools (Figure 6.1).

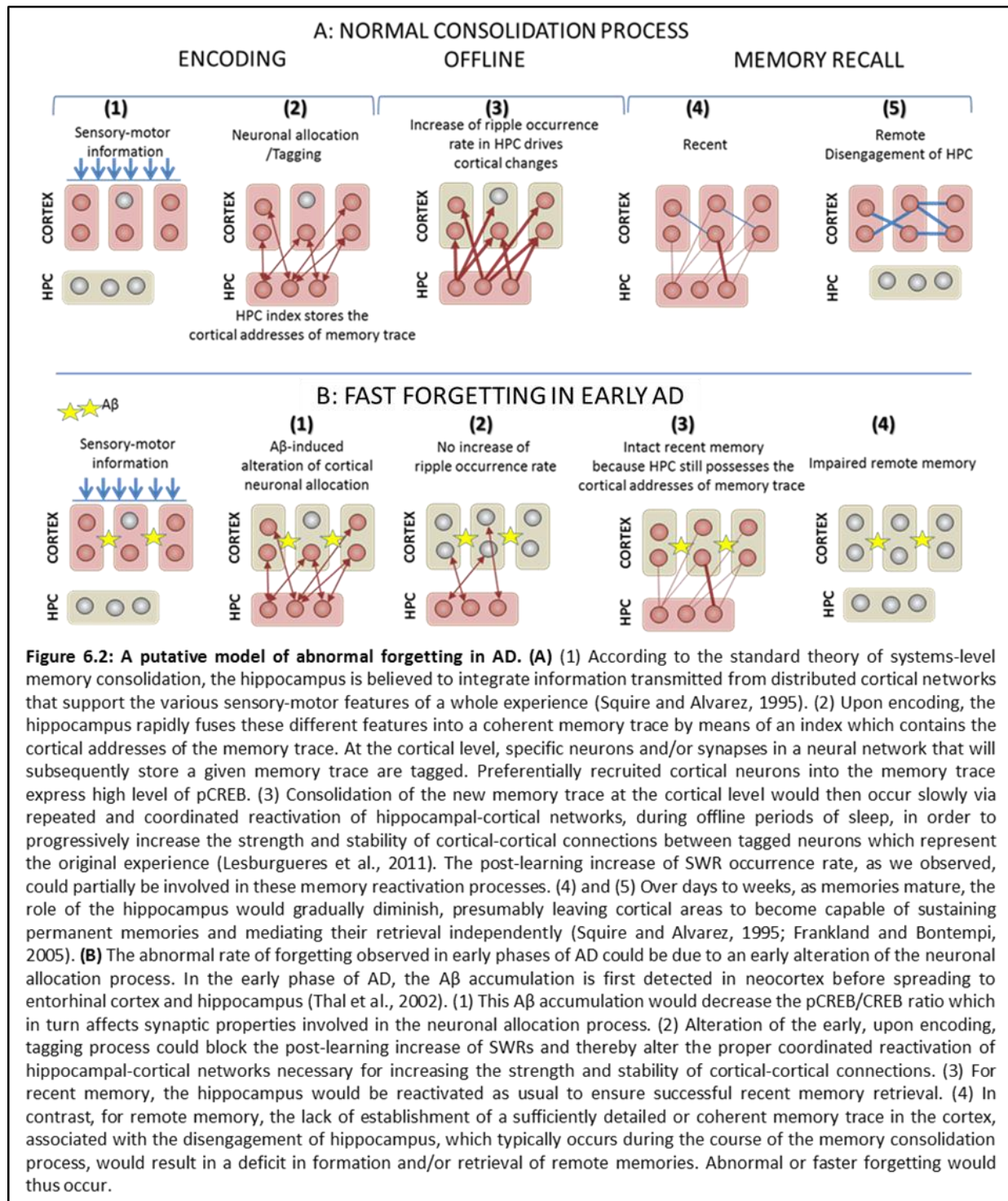
For instance, the Laurent Groc's team, at the Interdisciplinary Institute for Neuroscience in Bordeaux, has developed competing ligands mimicking the last 15 amino acids of Glu2A or GluN2B carboxyl-terminal domain which efficiently displace surface NMDAR subtypes in hippocampal neurons (Bard et al., 2010). In addition, antibodies have also been used to specifically reduce the surface dynamics of NMDARs both *in vitro* and *in vivo* (Groc et al., 2006; Dupuis et al., 2014). These innovative tools, designed and probed to alter the surface dynamics of GluN2A- or GluN2B-containing NMDARs, could give us the unique opportunity to alter the receptor dynamics and distribution in neurons. It is important to note that most of our understanding of NMDAR function in cognitive processes is based on the use of pharmacological and molecular tools which only block the NMDAR activity and therefore do not discriminate the differential contributions of receptor location and mobility. Exploring the role of NMDARs in cognitive function using tools that do not target the channel function but its trafficking is simply necessary for testing the emerging and non-canonical role of NMDAR dynamics in key brain functions. Restoring the balance between synaptic and extrasynaptic NMDARs in AD by using these tools could constitute a

therapeutic opportunity to alleviate memory impairments by modulating NMDAR function without the side effects.

Human studies have provided supportive evidence that AD could be a disorder of rapid forgetting (Hart et al., 1988; Salmon et al., 1989; Carlesimo et al., 1993; Reed et al., 1998; Ally et al., 2013). This abnormal forgetfulness can be reliably pinpointed in early phases of AD using tests of delayed recall (Welsh et al., 1991) and can be detectable prior to the onset of AD. As previously observed in another mouse model of AD (Daumas et al., 2008), we provided converging evidence that both APP<sup>swe</sup>/PS1<sup>dE9</sup> mice and A $\beta$ -injected mice exhibited faster forgetting compared to controls, a memory profile pointing to an inability to form and stabilize, or retrieve, long-lasting memories. We found that the accelerated memory decay of AD mice was associated with an abolishment of the two time-limited peaks of SWRs normally seen in controls, suggesting that their occurrence likely constitutes a prerequisite for the formation and accurate expression of spatial recognition memory. Although many cellular and synaptic mechanisms can explain the A $\beta$ -induced lack of SWRs triggering upon a cognitive challenge, one putative mechanism has been recently proposed to result from NMDAR plasticity and early neuronal allocation (i.e. “tagging”) of hippocampal-cortical networks (Lesburgueres et al., 2011; Girardeau et al., 2014).

Indeed the allocation of memory to specific neurons (neuronal allocation) and synapses (synaptic allocation) in a neurocircuit is not random, but rather dependent on specific mechanisms, which determine the exact sites where memories are stored (Rogerson et al., 2014). Thus, deficits of memory allocation could lead to blockade of the memory consolidation process and a faster forgetting as observed by pharmacological inactivation of NMDAR in specific cortical regions (Lesburgueres et al., 2011). Among the mechanisms which control the neuronal allocation, elegant studies have shown that neurons with relatively higher CREB function were more likely to be included into a given neuronal network, whereas neurons with relatively lower CREB function were more likely to be excluded from the memory trace (Han et al., 2007; Han et al., 2009; Zhou et al., 2009). However, studies performed in animal models of AD have shown that soluble A $\beta$  impaired the CREB signaling pathway (Vitolo et al., 2002; DaRocha-Souto et al., 2012) via the recruitment of extrasynaptic NMDARs (Li et al., 2011). Taken together, our study and others suggest that A $\beta$  via the extrasynaptic NMDAR activation (related to the mislocation of

NMDAR and/or the alteration of glutamate uptake) could reduce CREB signaling pathways. The deficits of memory allocation related to the CREB signaling pathway impairment induced by A $\beta$  could lead to an abolishment of SWRs and consequently an inability to form long-lasting memories (Figure 6.2). In contrast over-expression of CREB-binding protein or CREB could restore the memory allocation process and rescue memory deficits as shown in a transgenic mouse model of AD (Sato et al., 2009; Caccamo et al., 2010).



Advancing our understanding of these mechanisms in pathological conditions will help design better therapeutics to restore NMDAR balance by 1) decreasing extracellular glutamate spillover/release and tonic NMDAR activation, or 2) selectively blocking extrasynaptic NMDARs or their downstream signaling, or 3) enhancing cell-survival NMDAR signaling.



## B. PERSPECTIVES

Our current findings identify the disorganization of NMDAR subunits at the synapse as a potentially crucial mechanism underlying memory dysfunction in pathological processes such as those triggered in AD. However, because an array of deleterious mechanisms is triggered either simultaneously or in parallel in transgenic models of AD, additional experiments are required to demonstrate the functional relevance and properties of such a mechanism in a more controlled manner by using adult mice. To achieve this goal, experiments should be carried out and consist in displacing subunit specific-NMDARs in or out of synapses by using specific NMDAR modulators and determining the outcome on memory performance in adult C57BL/6 mice. Two complementary strategies could be used to interfere with the NMDAR location at the synapse: 1/ Mimicking of the PSD-95 protein downregulation in the hippocampus of AD mice by injecting into this region lentiviral vectors which express shRNA against PSD-95; 2/ Testing the impact of novel competing ligands mimicking the last 15 amino acids of Glu2A or GluN2B carboxyl-terminal domain which efficiently displace surface NMDAR subtypes (Bard et al., 2010). These innovative biomimetic peptides would give the unique opportunity to alter specifically the surface dynamics of GluN2-NMDARs in hippocampal neurons of behaving mice.

The impact of these manipulations on cognitive performance could be examined using a spatial discrimination paradigm in the Y-Maze, for which we demonstrated the high throughput potential for detecting subtle changes in memory functions and the high sensitivity to extrasynaptic NMDAR activation. The ability of the NMDAR modulators to effectively destabilize the GluN2B location in cognitively challenged animals could be confirmed by subcellular fractionation of hippocampal tissue. These data could help elucidating some of the underlying cellular and molecular mechanisms for cognitive dysfunction associated with AD and identifying novel therapeutic targets and tools for promoting recovery at early stages of the disease.

In order to further evaluate the deleterious role of A $\beta$  in neuronal allocation, we could use the social transmission of food preference (STFP) task in which rats learn about the safety of potential food sources by sampling those sources on the breath of conspecifics. This task enables olfactory information to be encoded rapidly during one single interaction

session and induces a memory which is robust and long-lasting. Its associative nature requires the hippocampus and specific cortical regions such as the orbitofrontal (OFC) and anterior cingulate cortices which are involved in processing olfactory associative information. Using this task, Lesburguères et al (2011) have shown that specific neurons or even synapses in the orbitofrontal cortex are presumably “tagged” and thereby allocated to participate in a memory for a particular food odor. In the STFP task, memories become increasingly dependent on the OFC as they progressively mature and are being stored at the cortical level. Thus, remote memory retrieval assessed 30 days after the social interaction experience is dependent on the OFC while recent memory retrieval (24 hour delay) is not. This behavioral task could give us the opportunity to further validate our working model in which we postulate that the early cortical accumulation of A $\beta$  peptides, as observed in the early phase of AD, by affecting glutamatergic neurotransmission (GluN2B mislocation or/and glutamate reuptake inhibition), would lead to a reduction of the activity of CREB and affect the neuronal allocation process. In contrast to recent memory, remote memory would be predominantly impaired, a finding that would explain the faster forgetting observed in early AD. We could first evaluate the impact of intra-OFC A $\beta$  accumulation on recent and remote memory, and next determine the pCREB/CREB ratio and also the subcellular distribution of GluN2B-containing NMDAR subunits. Finally, we could also determine the impact of the glutamate uptake inhibitor, DL-TBOA on pCREB/CREB ratio and further on recent and remote memory measured in the STFP task. In parallel, we could examine the impact of neuronal tagging alteration induced by either A $\beta$  or DL-TBOA intra-OFC infusions on post-learning SWR occurrence rates which we have shown to be specifically triggered by encoding and recognition processes.

## VII. ANNEXES

## Annex 1. Differential roles of GluN2A and/or GluN2B in memory processes (1/2).

Memory processes	Species	Age	Tissue	Manipulations	Behavioral Test	Involved NMDAR subunit	Reference
Encoding/reversal	Mouse	>P150	Forebrain	GluN2B <sup>-/-</sup> (forebrain)	Morris Water Maze	-	Engelhardt et al., 2008
			Hippocampus	GluN2B (hippocampus)	Morris Water Maze		
Learning	Mouse	2-3 month	All	GluN2A <sup>-/-</sup>	Elevated T-maze/radial maze	GluN2A	Bannerman et al., 2008
				CTD of GluN2A, GluN2A-CTD <sup>-/-</sup>		No role of GluN2A	
				GluN2A <sup>-/-</sup>	Morris water maze		
Learning	Mouse	?	All	GluN2A <sup>-/-</sup>	Morris water maze	GluN2A	Sakimura et al., 1995
Encoding	Rat	?	Amygdala, LBA	APV, freely moving, 0.5µg	Fear conditioning	-	Bauer et al., 2002
Reconsolidation	Rat	?	Amygdala, LBA	Ifenprodil (1µg/0.25µl); APV (2.5µg/0.5µl)	Fear conditioning	-	Ben Mamou et al., 2006
Learning	Mouse	12 weeks	dCA1 and cortex	GluN2B <sup>-/-</sup> in CaMKII neurons	Morris Water Maze	GluN2B	Brigman et al., 2010
				GluN2A <sup>-/-</sup>	Appetitive pairwise visual discrimination	GluN2A	
Learning	Mouse	14-18 month	Forebrain	Overexpression GluN2B (Forebrain)	Novel object recognition	GluN2B	Cao et al., 2007
					Fear conditioning		
					T-Maze		
					Morris Water Maze		
Learning	Rat	4-24 month	Hippocampus	Aging shRNA	Morris Water Maze	-	Clayton et al., 2002
Encoding/Reversal	Rat		Hippocampus	Vorinostat (increases histone acetylation)	Fear conditioning	GluN2B	Fujita et al., 2012
					Novel object recognition		
Pattern separation	Mouse			GluN2A knock-out mice	Similar object but change distance	GluN2A	Kannangara et al., 2014
Encoding	Mouse	3 and 12 month		Ro 25-6981 (10mg/Kg)	Contextual fear conditioning	GluN2B	Mathur et al., 2009
Encoding	Mouse/Rat		ACC HPC	Ro 25-6981 (2µg/0.5µl/side)	Contextual fear conditioning	GluN2B No role of GluN2B	Zhao et al., 2005
Remote memory retention	Rat	9 weeks 10 weeks	PFC PFC	APV APV	Trace eyeblink conditioning (periobital shock)	Role of NMDAR	Takehara-Nishiuchi et al., 2006
					Trace eyeblink conditioning (periobital shock)		
Extinction	Rat	10-12 weeks	PFC	Corticosterone (50µg/ml) in drinking water	Fear conditioning	GluN2B	Gourley et al., 2009
Reconsolidation	Rat		LBA	Ifenprodil	Fear conditioning	GluN2B	Ben Mamou et al., 2006

## Annex 1. Differential roles of GluN2A and/or GluN2B in memory processes (2/2).

Memory processes	Species	Age	Tissue	Manipulations	Behavioral Test	Subunit Role	Reference
Remote memory	Mouse			RO25-6981	Fear conditioning	GluN2B	Corcoran et al., 2013
Encoding	Mouse		HPC	RO25-6981/Ifenprodil	Fear conditioning	GluN2B	Descaizi et al., 2012
			ACC	RO25-6981 (1µg)			
Reconsolidation (destabilization)	Rat		BLA	Ifenprodil (1µg side)	Fear conditioning	GluN2B	Milton et al., 2013
				NVP-AAM077(2.5µg side)		GluN2A	
Extinction	Rat			RO25-6981 (6mg/kg)	Fear conditioning	GluN2B	Dalton et al., 2012
Encoding				NVP-AAM077(1.2mg/kg)		GluN2A	
Reversal	Rat			RO25-6981 (6mg/kg)	Operant chamber	GluN2B	Dalton et al., 2012
Encoding Recall	Rat		Amygdala	Ifenprodil	Fear conditioning	GluN2B	Rodrigues et al., 2001
						No role of GluN2B	
Encoding Recall	Rat		Amygdala	CP101,606 (0.5-4.5µg/amygdala) CP101,606 (0.5-4.5µg/amygdala)	Fear conditioning	GluN2A and GluN2B	Walker et al., 2012
						GluN2A	
Recall recent and remote memory	Mouse	9 wks	RSC	APV (10µg/µl: 0.16µl)	Fear conditioning	GluN2A	Corcoran et al., 2011
Encoding	Mouse	9 wks	Hippocampus	NVP (0.25-0.5µg); Ro25-6981 (0.5-2.5µg)	Fear conditioning	GluN2A and GluN2B	Gao et al., 2010
Extinction	Rat		BLA	Ifenprodil	Fear conditioning	GluN2B	Store-Bayon et al., 2007
recall	Rat		Hippocampus	APV	Cheeseboard (paired-associated learning)	GluN2B	Day et al., 2003
Encoding	Rat			APV	Morris water maze	Role of NMDAR	Morris et al., 1986
Forgetting	Rat			CPP	8-arm radial maze	Role of NMDAR	Villarreal et al., 2001
Tagging	Rat		OFC	APV	Social transmission of food preference	Role of NMDAR	Lesburguères et al., 2011
Slow Wave Sleep Ripple generation	Rat		Hippocampus	MK801	8-arm radial maze	Role of NMDAR	Girardes et al., 2015

## Annex 2: Behavioral and pathological characterizations of APPsw/PS1dE9 (1/2).

Reference	Age	Gender	Cognitive state	Pathological characteristic
1 Savonenko et al., 2005	6 months	male	MWM, RR, 6RWMT: no cognitive impairment	visible plaque deposition
	18 months	male	MWM, RR, 6RWMT: cognitive impairment	increased amyloid burden decreased cholinergic markers (CX and HPC) decreased stomatostatin
2 Melnikova et al., 2006	12 months	male	3-trial Y-maze, OF: no cognitive impairment	-
	8 months	female	3-trial Y-maze: no cognitive impairment OF: hyperactivity	
3 Hooijmans et al., 2009	8 months	male	OF: hyperactivity MWM: no cognitive impairment RR, 12 CHB: cognitive impairment	visible plaque deposition in DG, CG, CA1, CA3 rCBV: normal no atrophy
	15 months	male	OF: hyperactivity MWM, 12 CHB: cognitive impairment RR: no cognitive impairment	same pattern of amyloid plaques but increased quantity rCBV: normal 15% thinner outer molecular layer of DG
4 O'Leary and Brown, 2009	16 months	male	BM: cognitive impairment	
5 Ramirez-Lugo et al., 2009	3-4 months	male	CTA: normal	-
	7-8 months		CTA: impairment	
	15-16 months		CTA: impairment	
6 Kilgore et al., 2010	4 months	male	CFC: no cognitive impairment	-
	6 months		CFC: cognitive impairment	
7 Timmer et al., 2010	5 months	male	OF: hyperactivity MWM: cognitive impairment	increased Aβ1-40 and Aβ1-42 brain levels
	12 months	male	OF: no cognitive impairment MWM: cognitive impairment	
8 Bonardi et al., 2011	4 months	female	T-maze; OF; OLT; ORT; CFC: no cognitive impairment	
9 Zhang et al., 2011	12 months	male	MWM; SDPAT-retention: cognitive impairment	numerous plaque deposition increased Aβ1-42 to Aβ1-40
10 Xiong et al., 2011	6 months	male	MWM: cognitive impairment	Aβ1-40/Aβ1-42 1:10
	12 months	male	MWM: cognitive impairment	increased α-/β-secretase activity decreased γ-secretase activity blood Aβ levels stable from 6 to 12 months
11 Zhang et al., 2011	12 months	male	MWM: cognitive impairment	-
12 Lim et al., 2012	10 months	male	T-maze; OF; EPM; MWM: no cognitive impairment SBT: impairment	-
13 Zhang et al., 2012a	2.5 months	female	MWM: no cognitive impairment	no plaques normal levels of Ach and ChAT normal oxidative stress markers
	3.5 months	female	MWM: cognitive impairment	no plaques but increased sAβ compared to 2.5 months decreased levels of Ach and ChAT decreased SOD, GSH-px, increased MDA
14 Xue et al., 2012	8 months	female	MWM: cognitive impairment	-
15 Yang et al., 2012	8 months	female	MWM: cognitive impairment	numerous plaque deposition
16 Sierksma et al., 2013	5 months	male	Y-maze; OLT (ITI 24h): cognitive impairment FST; OLT (ITI 1h and 4h): no cognitive impairment	-
17 Laursen et al., 2013	8 months	male	T-maze: no cognitive impairment	visible plaque deposition normal ChAT activity
	4 months	male	OF; MWM: no cognitive impairment	-
18 Wang et al., 2013	6 months	male	OF: hyperactivity; MWM: cognitive impairment	deficient energy metabolism (glucose uptake PET-18F-FDG) decreased GSK-3β phosphorylation
	6-7 months	male	MWM; STT-retention: cognitive impairment	visible plaque deposition, increased levels of APP in HPC and CX eccentrically dispersed nuclei and swollen neuronal bodies decreased SOD, GSH-px, increased MDA increased JNK2, p53 and cleaved caspase 3
20 Toth et al., 2013	7 months	male	OF: no cognitive impairment MWM; BM: cognitive impairment	visible plaque deposition increased number of apoptotic cells and degraded neurons in HPC and CX
21 Vollert et al., 2013	15 months	male	OF: no cognitive impairment MWM: cognitive impairment	-
22 Rantamaki et al., 2013	3 months	female	-	decreased BDNF
	7 months	female		normal levels of BDNF
	12 months	female		increasing levels of BDNF in HPC and cortex from 12 to 15 months normal NGF
	12 months	male		visible plaque deposition
23 Bennett et al., 2013	2 months	male	MWM: no cognitive impairment	increased plaque deposition from 2 to 9 months
	9 months	male	MWM; BM: no cognitive impairment	astrocytosis
24 Ramos-Rodriguez et al., 2013	7 months	male	MWM: cognitive impairment	visible plaque deposition presence of tau phosphorylation in HPC and CX
25 Sierksma et al., 2014	5 months	male	OF: hyperactivity Y-maze; FST: no cognitive impairment OLT; Nest: cognitive impairment	normal level of BDNF, synaptophysin and PSD-95 decreased pCREB normal GSK-3β (Ser9) and tau Ser396, increased total tau levels
26 Curris et al., 2014	9 months	male	MWM: cognitive impairment	astrocytosis increased cytosolic phospholipase A2 increased COX1 (possible due microgliosis) increased proinflammatory thromboxanes 1 and 2 increased toll-like receptors
	12 months	male	MWM; EPM: cognitive impairment	-

## Annex 2: Behavioral and pathological characterizations of APPswe/PS1dE9 (2/2).

Reference	Age	Gender	Cognitive state	Pathological characteristic	
27	Koivisto et al., 2014	13 months	male	OF: hyperactivity MWM; ORT: cognitive impairment	increased $\beta$ -secretase activity, normal $\gamma$ -secretase activity increased FOX1 levels in HC, normal in cerebellum microgliosis
28	Izco et al., 2014	3-4 months	male		sporadic amyloid depositions in HPC & CX
		6 months	male	MWM: cognitive impairment progressing with age	increasing plaques loads with age
		8 months			increasing microgliosis with age (3 to 12 months)
12 months		decreasing A $\beta$ 1-40 and A $\beta$ 1-42 plasma levels with age			
29	Xu et al., 2014	8 months	male	MWM: cognitive impairment	astrocytosis and microgliosis
30	García et al., 2014	6 months	male	EPM; OF: no cognitive impairment SRT: cognitive impairment	increasing plaques loads from 6 to 12 months decreasing microvessels number from 6 to 12 months
		9-12 months	male	SRT; EPM: cognitive impairment	increasing astrocytosis and microgliosis from 9 to 12 months
31	Pedros et al., 2014	3 months	male	ORT: no cognitive impairment	decreased mRNA expression of InsR and InsR sub2 decreased mRNA expression of AMP-activated prot.kinase $\alpha$ 2 isoform decreased mRNA expression of PGC-1 $\alpha$
		6 months	male	ORT: cognitive impairment	increased p-tau, normal levels of synaptophysin and PSD-95 increased ILG factor 2 and ILGF binding protein 2 increased p-tau, normal levels of synaptophysin and PSD-95
32	Wang et al., 2014	7 months	both	ORT; MWM: cognitive impairment	visible amyloid plaques mitochondrial dysfunction
33	Zhou et al., 2014	8 months	female	MWM: cognitive impairment	robust amyloid plaques astrocytosis and microgliosis decreased SOD, GSH-px, increased MDA
34	Vegh et al., 2014	3 months	male	CFC: cognitive impairment MWM: no cognitive impairment	no amyloid plaques normal levels of PSD-95, AMPAR, NMDAR
		6 months			few visible amyloid plaques
		12 months			increased amyloid plaques
35	Ramos-Rodriguez et al., 2014	6 months	male	MWM: cognitive impairment	visible amyloid plaques, increased p-tau, microgliosis
36	Jiang et al., 2014	9 months	male	MWM: cognitive impairment	-
37	Edwards et al., 2014	3.5 months	male	MWM: cognitive impairment progressing with age	-
		9 months			
		15 months			
38	Tan et al., 2014	6 months	male	MWM: cognitive impairment	increased NLRP1, slightly reduced NeuN
		9 months			amyloid plaques progressing with age
39	Kemppainen et al., 2014	3.5 months	male	OF: hyperactivity; MWM: cognitive impairment	microgliosis
40	Li et al., 2014	7 months	male	MWM: cognitive impairment	-
		15 months	male	MWM: cognitive impairment	decreased glutamatergic neurotransmission
		22 months	male	ORT (ITI 10 min): cognitive impairment	decreased number of synapses, decreased synaptophysin decreased GABAergic terminals
41	Yanagisawa et al., 2015	14.5 months	male	Y-maze; MWM: cognitive impairment	astrocytosis and microgliosis
42	Gao et al. 2015	5 months	male	MWM: cognitive impairment	microgliosis and decreased SOD activity
43	Petrov et al., 2015	6 months	male	ORT: cognitive impairment	increased phosphorylated tau Ser 404
44	Janus et al., 2015	8&13 months	both	CFC: cognitive impairment	amyloid plaques progressing with age
		11&17 months	both	MWM: cognitive impairment	
		12&18 months	both	Burrowing and nest: impaired	

**MWM**-Morris water maze; **RR**-repeated reversal task; **OF**-open field; **6RWMT**-6-arm radial water maze task; **12 CHB**-12-circular hole board; **BM**-Barnes maze; **CTA**-conditioned taste aversion; **CFC**-contextual fear conditioning; **OLT**-object location task; **ORT**-object recognition task; **SDPAT**-step down type passive avoidance test; **EPM**-elevated plus maze; **SBT**-stationary beam test; **FST**-forced swim test; **STT**-step through test; **SRT**-social recognition test; **CT**-Cortex; **HPC**-Hippocampus; **DG**-dentate gyrus; **CG**-cingulate gyrus; **rCBV**-relative cerebral blood volume; **Ach**-acetylcholine; **ChAT**-choline acetyltransferase; **SOD**-superoxide dismutase; **GSH-px**-glutathione peroxidase; **MDA**-malondialdehyde; **GSK-3 $\beta$** -glycogen synthase kinase 3 beta; **BDNF**-brain-derived neurotrophic factor; **NGF**-nerve growth factor; **pCREB**-phosphorylated cAMP response element-binding protein; **COX1**-cyclooxygenase 1; **InsR**-insulin receptor; **ILGF**-insulin like growth factor; **PGC-1 $\alpha$** -peroxisome proliferator-activated receptor gamma coactivator 1-alpha; **NLRP1**-NOD-like receptor family, pyrin domain containing 1; **OFc**-orbitofrontal cortex; **ACC**-anterior cingulate cortex; **BLA**-basolateral amygdala.

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